



Europäisches Patentamt  
European Patent Office  
Office européen des brevets

Publication number:

0 272 009  
A2

(2)

## EUROPEAN PATENT APPLICATION

(2) Application number: 87310363.4

(51) Int. Cl.: C12Q 1/68 , G01N 33/53 ,  
C12Q 1/04 , C12Q 1/00 ,  
C07H 21/00 , //C12P19/34

(2) Date of filing: 24.11.87

(3) Priority: 24.11.86 US 934244  
07.08.87 US 83542

(7) Applicant: Hogan, James John  
2727 First Avenue  
San Diego California 92103(US)

(3) Date of publication of application:  
22.06.88 Bulletin 88/25

Applicant: Smith, Richard Dana  
3522 Mercer Lane  
San Diego California 92122(US)

(3) Designated Contracting States:  
AT BE CH DE ES FR GB GR IT LU NL SE

Applicant: Kop, Jo Ann  
1314 Hampton Road  
San Marcos California 92069(US)

Applicant: McDonough, Sherrol Hoffa  
4697 Robbins Street  
San Diego California 92122(US)

(7) Inventor: Hogan, James John  
2727 First Avenue  
San Diego California 92103(US)  
Inventor: Smith, Richard Dana  
3522 Mercer Lane  
San Diego California 92122(US)  
Inventor: Kop, Jo Ann  
1314 Hampton Road  
San Marcos California 92069(US)  
Inventor: McDonough, Sherrol Hoffa  
4697 Robbins Street  
San Diego California 92122(US)

(7) Representative: Goldin, Douglas Michael et al  
J.A. KEMP & CO. 14, South Square Gray's Inn  
London WC1R 6EU(GB)

EP 0 272 009 A2

(3) Nucleic acid probes for detection and/or quantitation of non-viral organisms.

(57) A method for preparing probes, as well as several probes for use in qualitative or quantitative hybridization assays are disclosed. The method comprises constructing an oligonucleotide that is sufficiently complementary to hybridize to a region of rRNA selected to be unique to a non-viral organism or group of non-viral organisms sought to be detected, said region of rRNA being selected by comparing one or more variable region rRNA sequences of said non-viral organism or group of non-viral organisms with one or more variable region rRNA sequences from one or more non-viral organisms sought to be distinguished. Hybridization assay probes for

Mycobacterium avium, Mycobacterium intracellulare, the Mycobacterium tuberculosis-complex bacteria, Mycoplasma pneumoniae, Legionella, Salmonella, Chlamydia trachomatis, Campylobacter, Proteus mirabilis, Enterococcus, Enterobacter cloacae, E. coli, Pseudomonas group I, Neisseria gonorrhoeae, bacteria, and fungi also are disclosed.

Mycobacterium avium, Mycobacterium intracellulare, the Mycobacterium tuberculosis-complex bacteria, Mycoplasma pneumoniae, Legionella, Salmonella, Chlamydia trachomatis, Campylobacter, Proteus mirabilis, Enterococcus, Enterobacter cloacae, E. coli, Pseudomonas group I, Neisseria gonorrhoeae, bacteria, and fungi also are disclosed.

## NUCLEIC ACID PROBES FOR DETECTION AND/OR QUANTITATION OF NON-VIRAL ORGANISMS

BACKGROUND OF THE INVENTION1. Field of the Invention

5

The inventions described and claimed herein relate to probes and assays based on the use of genetic material such as RNA. More particularly, the inventions relate to the design and construction of nucleic acid probes and hybridization of such probes to genetic material of target non-viral organisms in assays for detection and/or quantitation thereof in test samples of, e.g., sputum, urine, blood and tissue sections, food, 10 soil and water.

2. Introduction

15 Two single strands of nucleic acid, comprised of nucleotides, may associate ("hybridize") to form a double helical structure in which the two polynucleotide chains running in opposite directions are held together by hydrogen bonds (a weak form of chemical bond) between pairs of matched, centrally located compounds known as "bases." Generally, in the double helical structure of nucleic acids, for example, the base adenine (A) is hydrogen bonded to the base thymine (T) or uracil (U) while the base guanine (G) is 20 hydrogen bonded to the base cytosine (C). At any point along the chain, therefore, one may find the base pairs AT or AU, TA or UA, GC, or CG. One may also find AG and GU base pairs in addition to the traditional ("canonical") base pairs. Assuming that a first single strand of nucleic acid is sufficiently complementary to a second and that the two are brought together under conditions which will promote their hybridization, double stranded nucleic acid will result. Under appropriate conditions, DNA/DNA, RNA/DNA, 25 or RNA/RNA hybrids may be formed.

Broadly, there are two basic nucleic acid hybridization procedures. In one, known as "in solution" hybridization, both a "probe" nucleic acid sequence and nucleic acid molecules from a test sample are free in solution. In the other method, the sample nucleic acid is usually immobilized on a solid support and the probe sequence is free in solution.

30 A probe may be a single strand nucleic acid sequence which is complementary in some particular degree to the nucleic acid sequences sought to be detected ("target sequences"). It may also be labelled. A background description of the use of nucleic acid hybridization as a procedure for the detection of particular nucleic acid sequences is described in U.S. Application Serial No. 456,729, entitled "Method for Detection, Identification and Quantitation of Non-Viral Organisms," filed January 10, 1983 (Kohne I), and 35 U.S. Application Serial No. 655,385, entitled "Method For Detecting, Identifying and Quantitating Organisms and Viruses," filed September 4, 1984 (Kohne II), both of which are incorporated by reference, together with all other applications cited herein.

Also described in those applications are methods for determining the presence of RNA-containing organisms in a sample which might contain such organisms, comprising the steps of bringing together any 40 nucleic acids from a sample and a probe comprising nucleic acid molecules which are shorter than the rRNA subunit sequence from which it was derived and which are sufficiently complementary to hybridize to the rRNA of one or more non-viral organisms or groups of non-viral organisms, incubating the mixture under specified hybridization conditions, and assaying the resulting mixture for hybridization of the probe and any 45 test sample rRNA. The invention is described to include using a probe which detects only rRNA subunit subsequences which are the same or sufficiently similar in particular organisms or groups of organisms and is said to detect the presence or absence of any one or more of those particular organisms in a sample, even in the presence of many non-related organisms.

We have discovered and describe herein a novel method and means for designing and constructing 50 DNA probes for use in detecting unique rRNA sequences in an assay for the detection and/or quantitation of any group of non-viral organisms. Some of the inventive probes herein maybe used to detect and/or quantify a single species or strain of non-viral organism and others may be used to detect and/or quantify members of an entire genus or desired phylogenetic grouping.

SUMMARY OF THE INVENTION

In a method of probe preparation and use, a single strand deoxyoligonucleotide of particular sequence and defined length is used in a hybridization assay to determine the presence or amount of rRNA from particular target non-viral organisms to distinguish them from their known closest phylogenetic neighbors. Probe sequences which are specific, respectively, for 16S rRNA variable subsequences of Mycobacterium avium, Mycobacterium intracellulare and the Mycobacterium tuberculosis-complex bacteria, and which do not cross react with nucleic acids from each other, or any other bacterial species or respiratory infectious agent, are described and claimed. A probe specific to a 23S rRNA variable region 10 subsequence from the Mycobacterium tuberculosis-complex bacteria is also described and claimed, as are rRNA variable region probes useful in hybridization assays for the genus Mycobacterium (23 s rRNA specific), Mycoplasma pneumoniae (5S and 16S rRNA-specific), Chlamydia trachomatis (16S and 23S rRNA specific), Enterobacter cloacae (23S rRNA specific), Escherichia coli (16S rRNA specific), Legionella (16S and 23S rRNA specific), Salmonella (16S and 23S rRNA specific), Enterococci (16S rRNA specific), Neisseria gonorrhoeae (16s rRNA specific), Campylobacter (16S rRNA specific), Proteus mirabilis (23S rRNA specific), Pseudomonas (23S rRNA specific), fungi (18S and 28S rRNA specific), and bacteria (16S and 23S rRNA specific).

In one embodiment of the assay method, a test sample is first subjected to conditions which release rRNA from any non-viral organisms present in that sample. rRNA is single stranded and therefore available 20 for hybridization with sufficiently complementary genetic material once so released. Contact between a probe, which can be labelled, and the rRNA target may be carried out in solution under conditions which promote hybridization between the two strands. The reaction mixture is then assayed for the presence of hybridized probe. Numerous advantages of the present method for the detection of non-viral organisms over prior art techniques, including accuracy, simplicity, economy and speed will appear more fully from the 25 detailed description which follows.

BRIEF DESCRIPTION OF THE DRAWING

- 30 Figure 1 is a chart of the primary structure of bacterial 16S rRNA for Escherichia coli, depicting standard reference numbers for base pairs.
- Figure 2 is a chart of the primary structure of bacterial 23S rRNA for Escherichia coli, depicting standard reference numbers for base pairs.
- 35 Figure 3 is a chart of the primary structure of bacterial 5S rRNA for Escherichia coli, depicting standard reference numbers for base pairs.
- Figure 4 is a chart of the primary structure for the 18S rRNA for Saccharomyces cerevisiae, depicting standard reference numbers for base pairs.
- Figure 5 is a chart of the primary structure for the 28S rRNA for Saccharomyces cerevisiae, depicting standard reference numbers for base pairs.
- 40 Figure 6 is a diagram showing the locations in the 16S rRNA (using E. coli reference numbers) which differ between 12 different sets of related organisms. In Example 1, for example, 89.7 refers to the difference in 16S rRNA between Clostridium botulinum and Clostridium subterminale.
- Figure 7 is a diagram showing the locations in the first 1500 bases of 23S rRNA (using E.coli reference numbers ) which differ between 12 different sets of related organisms.
- 45 Figure 8 is a diagram showing the locations in the terminal bases of 23S rRNA (using E.coli reference numbers) which differ between 12 different sets of related organisms.
- Figure 9 is a schematic representation of the location of probes capable of hybridizing to the 16S rRNA.
- 50 Figure 10 is a schematic representation of the location of probes capable of hybridizing to the first 1500 bases of the 23S rRNA.
- Figure 11 is a schematic representation of the location of probes capable of hybridizing to the terminal bases of 23S rRNA.

55 DETAILED DESCRIPTION OF THE INVENTION

Definitions

The following terms, as used in this disclosure and claims, are defined as:

5

nucleotide:

a subunit of a nucleic acid consisting of a phosphate group, a 6' carbon sugar and a nitrogen containing base. In RNA the 5' carbon sugar is ribose. In DNA, it is a 2-deoxyribose. The term also includes analogs of such subunits.

nucleotide polymer:

15 at least two nucleotides linked by phosphodiester bonds.

oligonucleotide:

20 a nucleotide polymer generally about 10 to about 100 nucleotides in length, but which may be greater than 100 nucleotides in length.

nucleic acid probe:

25

a single stranded nucleic acid sequence that will combine with a complementary single stranded target nucleic acid sequence to form a double-stranded molecule (hybrid). A nucleic acid probe may be an oligonucleotide or a nucleotide polymer.

30

hybrid:

the complex formed between two single stranded nucleic acid sequences by Watson-Crick base pairings or non-canonical base pairings between the complementary bases.

35

hybridization:

the process by which two complementary strands of nucleic acids combine to form double stranded molecules (hybrids).

complementarity:

45

a property conferred by the base sequence of a single strand of DNA or RNA which may form a hybrid or double stranded DNA:DNA, RNA:RNA or DNA:RNA through hydrogen bonding between Watson-Crick base pairs on the respective strands. Adenine (A) usually complements thymine (T) or Uracil (U), while guanine (G) usually complements cytosine (C).

50

stringency:

55

term used to describe the temperature and solvent composition existing during hybridization and the subsequent processing steps. Under high stringency conditions only highly homologous nucleic acid hybrids will form; hybrids without a sufficient degree of complementarity will not form. Accordingly, the stringency of the assay conditions determine the amount of complementarity needed between two nucleic acid strands forming a hybrid. Stringency is chosen to maximize the difference in stability between the target and the nontarget.

probe specificity:

characteristic of a probe which describes its ability to distinguish between target and nontarget sequences. Dependent on sequence and assay conditions. Probe specificity may be absolute (i.e., probe able to distinguish between target organisms and any nontarget organisms), or it may be functional (i.e., probe able to distinguish between the target organism and any other organism normally present in a particular sample). Many probe sequences can be used for either broad or narrow specificity depending on the conditions of use.

10

variable region:

nucleotide polymer which differs by at least one base between the target organism and nontarget organisms contained in a sample.

15

conserved region:

a region which is not variable.

20

sequence divergence:

process by which nucleotide polymers become less similar during evolution.

25

sequence convergence:

process by which nucleotide polymers become more similar during evolution.

30

bacteria:

members of the phylogenetic group eubacteria, which is considered one of the three primary kingdoms.

35

Tm:

temperature at which 50% of the probe is converted from the hybridized to the unhybridized form.

40

thermal stability:

a condition of hybridization incubation or separation incubation in which stable probe:target hybrids will form and in which no probe:nontarget hybrids can form by virtue of their instability. Temperature at which 50% of the probe:target hybrids are converted to the single stranded form. Factors which affect the thermal stability can affect probe specificity and therefore, must be controlled. Whether a probe sequence is useful to detect only a specific type of organism depends largely on the thermal stability difference between probe:target hybrids ("P:T") and probe:nontarget hybrids ("P:NT"). In designing probes the Tm P:T minus the Tm P:NT should be as large as possible.

In addition to a novel method for selecting probe sequences, we have discovered that it is possible to create a DNA probe complementary to a particular rRNA sequence obtained from a single target microorganism and to successfully use that probe in a non-cross reacting assay for the detection of that single microorganism, even in the presence of its known, most closely related taxonomic or phylogenetic neighbors. With the exception of viruses, all prokaryotic organisms contain rRNA molecules including 5S rRNA, 16S rRNA, and a larger rRNA molecule known as 23S rRNA. Eukaryotes are known to have 5.0S, 5.8S, 18S and 28S rRNA molecules or analogous structures. (The term "16S like" sometimes is used to refer to the rRNA found in the small ribosomal subunit, including 18S and 17S rRNA. Likewise the term

"23S like" rRNA sometimes is used to refer to the rRNA found in the large ribosomal subunit. "5S like" rRNA sometimes is used to refer to rRNA found in the small ribosomal subunit. 5.8S rRNA is equivalent to the 5' end of the 23S like rRNA.) These rRNA molecules contain nucleotide sequences which are highly conserved among all organisms thus far examined. There are known methods which allow a significant portion of these rRNA sequences to be determined. For example, complementary oligonucleotide primers of about 20-30 bases in length can be hybridized to universally conserved regions in purified rRNA that are specific to the 5S, 16S, or 23S subunits and extended with the enzyme reverse transcriptase. Chemical degradation or dideoxynucleotide-terminated sequencing reactions can be used to determine the nucleotide sequence of the extended product. Lane, D.J. et al., Proc. Natl. Acad. Sci. USA 82, 6955-6959 (1985).

In our invention, comparison of one or more sequenced rRNA variable regions from a target organism to one or more rRNA variable region sequences from a closely related bacterial species is utilized to select a sequence unique to the rRNA of the target organism. rRNA is preferable to DNA as a probe target because of its relative abundance and stability in the cell and because of its patterns of phylogenetic conservation.

Notwithstanding the highly conserved nature of rRNA, we have discovered that a number of regions of the rRNA molecule which can vary in sequence, can vary even between closely related species and can, therefore, be utilized to distinguish between such organisms. Differences in the rRNA molecule are not distributed randomly across the entire molecule, but rather are clustered into specific regions. The degree of conservation also varies, creating a unique pattern of conservation across the ribosomal RNA subunits.

The degree of variation and the distribution thereof, can be analyzed to locate target sites for diagnostic probes. This method of probe selection may be used to select more than one sequence which is unique to the rRNA of a target organism.

We have identified variable regions by comparative analysis of rRNA sequences both published in the literature and sequences which we have determined ourselves using procedures known in the art. We use a Sun Microsystems (TM) computer for comparative analysis. The compiler is capable of manipulating many sequences of data at the same time. Computers of this type and computer programs which may be used or adapted for the purposes herein disclosed are commercially available.

Generally, only a few regions are useful for distinguishing between closely related species of a phylogenetically conserved genus, for example, the region 400-500 bases from the 5' end of the 16S rRNA molecule. An analysis of closely related organisms (Figures 6, 7 and 8) reveals the specific positions (variable regions) which vary between closely related organisms. These variable regions of rRNA molecules are the likely candidates for probe design.

Figures 5, 6 and 7 display the variations in 16S and 23S rRNA's between two different bacteria with decreasing amounts of similarity between them. Closer analysis of these figures reveals some subtle patterns between these closely related organisms. In all cases studied, we have seen sufficient variation between the target organism and the closest phylogenetic relative found in the same sample to design the probe of interest. Moreover, in all cases studied to date, the per cent similarity between the target organism (or organisms) and the closest phylogenetically related organisms found in the same sample has been between 90% and 99%. Interestingly, there was enough variation even between the rRNA's of Neisseria's gonorrhoeae and meningitidis (See Example 21) to design probes - despite the fact that DNA:DNA homology studies suggested these two species might actually be one and the same.

These figures also show that the differences are distributed across the entire 16S and 23S rRNA's. Many of the differences, nonetheless, cluster into a few regions. These locations in the rRNA are good candidates for probe design, with our current assay conditions. We also note that the locations of these increased variation densities usually are situated in the same regions of the 16S and 23S rRNA for comparable per cent similarity values. In this manner, we have observed that certain regions of the 16S and 23S rRNA are the most likely sites in which significant variation exists between the target organism and the closest phylogenetic relatives found in a sample. We have disclosed and claimed species specific probes which hybridize in these regions of significant variation between the target organism and the closest phylogenetic relative found in a sample.

Figures 9, 10 and 11 are a schematic representation of the location of probes disclosed and claimed herein. Because 16S and 23S RNAs do not, as a rule, contain sequences of duplication longer than about six nucleotides in length, probes designed by these methods are specific to one or a few positions on the target nucleic acid.

The sequence evolution at each of the variable regions (for example, spanning a minimum of 10 nucleotides) is, for the most part divergent, not convergent. Thus, we can confidently design probes based on a few rRNA sequences which differ between the target organism and its phylogenetically closest relatives. Biological and structural constraints on the rRNA molecule which maintain homologous primary,

secondary and tertiary structure throughout evolution, and the application of such constraints to probe diagnostics is the subject of ongoing study. The greater the evolutionary distance between organisms, the greater the number of variable regions which may be used to distinguish the organisms.

Once the variable regions are identified, the sequences are aligned to reveal areas of maximum homology or "match". At this point, the sequences are examined to identify potential probe regions. Two important objectives in designing a probe are to maximize homology to the target sequence(s) (greater than 90% homology is recommended) and to minimize homology to non-target sequence(s) (less than 90% non-homology to nontargets is recommended). We have identified the following useful guidelines for designing probes with desired characteristics.

10 First, probes should be positioned so as to minimize the stability of the probe:nontarget nucleic acid hybrid. This may be accomplished by minimizing the length of perfect complementarity to non-target organisms, avoiding G and C rich regions of homology to non-target sequences, and by positioning the probe to span as many destabilizing mismatches as possible (for example, dG:rU base pairs are less destabilizing than some others).

15 Second, the stability of the probe: target nucleic acid hybrid should be maximized. This may be accomplished by avoiding long A and T rich sequences, by terminating the hybrids with G:C base pairs and by designing the probe with an appropriate Tm. The beginning and end points of the probe should be chosen so that the length and %G and %C result in a TM about 2-10°C higher than the temperature at which the final assay will be performed. The importance and effect of various assay conditions will be 20 explained further herein. Third, regions of the rRNA which are known to form strong structures inhibitory to hybridization are less preferred. Finally, probes with extensive self-complementarity should be avoided.

In some cases, there may be several sequences from a particular region which will yield probes with the desired hybridization characteristics. In other cases, one sequence may be significantly better than another which differs merely by a single base.

25 The following chart indicates how, for one embodiment of the invention useful in the detection of a nucleic acid in the presence of closely related nucleic acid sequences, unique sequences can be selected. In this example, rRNA sequences have been determined for organisms A-E and their sequences, represented numerically, are aligned as shown. It is seen that sequence 1 is common to all organisms A-E. Sequences 2-6 are found only in organisms A, B and C, while sequences 8, 9 and 10 are unique to 30 organism A. Therefore, a probe complementary to sequences 8, 9 or 10 would specifically hybridize to organism A.

36 Illustrative Pattern of Sequence Relationships Among Related Bacteria

<u>Organism</u>	<u>rRNA Sequence</u>									
A	1	2	3	4	5	6	7	8	9	10
B	1	2	3	4	5	6	7	11	12	13
C	1	2	3	4	5	6	14	15	16	17
D	1	18	19	20	21	22	23	24	25	26
E	1	18	19	20	21	27	28	29	30	31

45 In cases where the patterns of variation of a macromolecule are known, for example, rRNA, one can focus on specific regions as likely candidates for probe design. However, it is not always necessary to determine the entire nucleic acid sequence in order to obtain a probe sequence. Extension from any single oligonucleotide primer can yield up to 300-400 bases of sequence. When a single primer is used to partially sequence the rRNA of the target organism and organisms closely related to the target, an alignment can be 50 made as outlined above. Plainly, if a useful probe sequence is found, it is not necessary to continue rRNA sequencing using other primers. If, on the other hand, no useful probe sequence is obtained from sequencing with a first primer, or if higher sensitivity is desired, other primers can be used to obtain more sequences. In those cases where patterns of variation for a molecule are not well understood, more sequence data may be required prior to probe design.

55 Thus, in Examples 1-3 below, two 16S-derived primers were used. The first primer did not yield probe sequences which met the criteria listed herein. The second primer yielded probe sequences which were determined to be useful following characterization and testing for specificity as described. In Example 4, six

23S primers were used prior to locating the probe sequence set forth.

Once a presumptive unique sequence has been identified, a complementary DNA oligonucleotide is synthesized. This single stranded oligonucleotide will serve as the probe in the DNA/rRNA assay hybridization reaction. Defined oligonucleotides may be synthesized by any of several well known methods, including

5 automated solid-phase chemical synthesis using cyanoethylphosphoramidite precursors. Barone, A.D. et al., Nucleic Acids Research 12, 4051-4060 (1984). In this method, deoxyoligonucleotides are synthesized on solid polymer supports. Release of the oligonucleotide from the support is accomplished by treatment with ammonium hydroxide at 60°C for 16 hours. The solution is dried and the crude product is dissolved in water and separated on polyacrylamide gels which generally may vary from 10-20% depending upon the  
10 length of the fragment. The major band, which is visualized by ultraviolet back lighting, is cut from the gel with a razor blade and extracted with 0.1M ammonium acetate, pH 7.0, at room temperature for 8-12 hours. Following centrifugation, the supernatant is filtered through a 0.4 micron filter and desalts on a P-10 column (Pharmacia). Other well known methods for construction of synthetic oligonucleotides may, of course, be employed.

15 Current DNA synthesizers can produce large amounts of synthetic DNA. After synthesis, the size of the newly made DNA is examined by gel filtration and molecules of varying size are generally detected. Some of these molecules represent abortive synthesis events which occur during the synthesis process. As part of post-synthesis purification, the synthetic DNA is usually size fractionated and only those molecules which are the proper length are kept. Thus, it is possible to obtain a population of synthetic DNA molecules of  
20 uniform size.

It has been generally assumed, however, that synthetic DNA is inherently composed of a uniform population of molecules all of the same size and base sequence, and that the hybridization characteristics of every molecule in the preparation should be the same. In reality, preparations of synthetic DNA molecules are heterogeneous and are composed of significant numbers of molecules which, although the  
25 same size, are in some way different from each other and have different hybridization characteristics. Even different preparations of the same sequence can sometimes have different hybridization characteristics.

Accordingly, preparations of the same synthetic probe sequence can have different hybridization characteristics. Because of this the specificity of probe molecules from different preparations can be different. The hybridization characteristics of each preparation should be examined in order to determine  
30 the hybridization conditions which must be used in order to obtain the desired probe specificity. For example, the synthetic probe described in Example 4 below has the specificity profile described in Table 14. This data was obtained by using the hybridization and assay conditions described. A separate preparation of this probe which has different hybridization characteristics may not have precisely the same specificity profile when assayed under the conditions presented in Example 4. Such probe preparations  
35 have been made. To obtain the desired specificity, these probes can be hybridized and assayed under different conditions, including salt concentration and/or temperature. The actual conditions under which the probe is to be used must be determined, or matched to extant requirements, for each batch of probe since the art of DNA synthesis is somewhat imperfect.

Following synthesis and purification of a particular oligonucleotide sequence, several procedures may  
40 be utilized to determine the acceptability of the final product. The first is polyacrylamide gel electrophoresis, which is used to determine size. The oligonucleotide is labelled using, for example,  $^{32}\text{P}$ -ATP and T<sub>4</sub> polynucleotide kinase. The labelled probe is precipitated in ethanol, centrifuged and the dried pellet resuspended in loading buffer (80% formamide, 20 mM NaOH, 1 mM EDTA, 0.1% Bromophenol blue and 0.1% xylene cyanol). The samples are heated for five minutes at 90°C and loaded onto a denaturing  
45 polyacrylamide gel. Electrophoresis is carried out in TBE buffer (0.1 M Tris HCl pH 8.3, 0.08 M boric acid, 0.002 M EDTA) for 1-2 hours at 1,000 volts. Following electrophoresis of the oligonucleotide the gel is exposed to X-ray film. The size of the oligonucleotide is then computed from the migration of oligonucleotide standards run concurrently.

The sequence of the synthetic oligonucleotide may also be checked by labelling it at the 5' end with  
50  $^{32}\text{P}$ -ATP and T<sub>4</sub> polynucleotide kinase, subjecting it to standard chemical degradation techniques, Maxam, A.M. and Gilbert, W., Proc. Nat'l. Acad. Sci. USA 74, 580-584 (1980), and analyzing the products on polyacrylamide gels. Preferably, the nucleotide sequence of the probe is perfectly complementary to the previously identified unique rRNA sequence, although it need not be.

The melting profile, including the melting temperature (T<sub>m</sub>) of the oligonucleotide/ rRNA hybrids should  
55 also be determined. One way to determine T<sub>m</sub> is to hybridize a  $^{32}\text{P}$ -labelled oligonucleotide to its complementary target nucleic acid at 50°C in 0.1 M phosphate buffer, pH 8.8. The hybridization mixture is diluted and passed over a 2cm hydroxyapatite column at 50°C. The column is washed with 0.1 M phosphate buffer, 0.02% SDS to elute all unhybridized, single-stranded probes. The column temperature is

23S primers were used prior to locating the probe sequence set forth.

Once a presumptive unique sequence has been identified, a complementary DNA oligonucleotide is synthesized. This single stranded oligonucleotide will serve as the probe in the DNA/rRNA assay hybridization reaction. Defined oligonucleotides may be synthesized by any of several well known methods, including 5 automated solid-phase chemical synthesis using cyanoethylphosphoramidite precursors. Barone, A.D. et al., Nucleic Acids Research 12, 4051-4080 (1984). In this method, deoxynucleotides are synthesized on solid-polymer supports. Release of the oligonucleotide from the support is accomplished by treatment with ammonium hydroxide at 60°C for 16 hours. The solution is dried and the crude product is dissolved in water and separated on polyacrylamide gels which generally may vary from 10-20% depending upon the 10 length of the fragment. The major band, which is visualized by ultraviolet back lighting, is cut from the gel with a razor blade and extracted with 0.1M ammonium acetate, pH 7.0, at room temperature for 8-12 hours. Following centrifugation, the supernatant is filtered through a 0.4 micron filter and desalts on a P-10 column (Pharmacia). Other well known methods for construction of synthetic oligonucleotides may, of course, be employed.

15 Current DNA synthesizers can produce large amounts of synthetic DNA. After synthesis, the size of the newly made DNA is examined by gel filtration and molecules of varying size are generally detected. Some of these molecules represent abortive synthesis events which occur during the synthesis process. As part of post-synthesis purification, the synthetic DNA is usually size fractionated and only those molecules which are the proper length are kept. Thus, it is possible to obtain a population of synthetic DNA molecules of 20 uniform size.

25 It has been generally assumed, however, that synthetic DNA is inherently composed of a uniform population of molecules all of the same size and base sequence, and that the hybridization characteristics of every molecule in the preparation should be the same. In reality, preparations of synthetic DNA molecules are heterogeneous and are composed of significant numbers of molecules which, although the same size, are in some way different from each other and have different hybridization characteristics. Even different preparations of the same sequence can sometimes have different hybridization characteristics.

Accordingly, preparations of the same synthetic probe sequence can have different hybridization characteristics. Because of this the specificity of probe molecules from different preparations can be different. The hybridization characteristics of each preparation should be examined in order to determine 30 the hybridization conditions which must be used in order to obtain the desired probe specificity. For example, the synthetic probe described in Example 4 below has the specificity profile described in Table 14. This data was obtained by using the hybridization and assay conditions described. A separate preparation of this probe which has different hybridization characteristics may not have precisely the same specificity profile when assayed under the conditions presented in Example 4. Such probe preparations 35 have been made. To obtain the desired specificity, these probes can be hybridized and assayed under different conditions, including salt concentration and/or temperature. The actual conditions under which the probe is to be used must be determined, or matched to extant requirements, for each batch of probe since the art of DNA synthesis is somewhat imperfect.

Following synthesis and purification of a particular oligonucleotide sequence, several procedures may 40 be utilized to determine the acceptability of the final product. The first is polyacrylamide gel electrophoresis, which is used to determine size. The oligonucleotide is labelled using, for example, <sup>32</sup>P-ATP and T<sub>4</sub> polynucleotide kinase. The labelled probe is precipitated in ethanol, centrifuged and the dried pellet resuspended in loading buffer (80% formamide, 20 mM NaOH, 1 mM EDTA, 0.1% Bromophenol blue and 0.1% xylene cyanol). The samples are heated for five minutes at 90°C and loaded onto a denaturing 45 polyacrylamide gel. Electrophoresis is carried out in TBE buffer (0.1 M Tris HCl pH 8.3, 0.08 M boric acid, 0.002 M EDTA) for 1-2 hours at 1,000 volts. Following electrophoresis of the oligonucleotide the gel is exposed to X-ray film. The size of the oligonucleotide is then computed from the migration of oligonucleotide standards run concurrently.

The sequence of the synthetic oligonucleotide may also be checked by labelling it at the 5' end with 50 <sup>32</sup>P-ATP and T<sub>4</sub> polynucleotide kinase, subjecting it to standard chemical degradation techniques, Maxam, A.M. and Gilbert, W., Proc. Nat'l. Acad. Sci. USA 74, 580-584 (1980), and analyzing the products on polyacrylamide gels. Preferably, the nucleotide sequence of the probe is perfectly complementary to the previously identified unique rRNA sequence, although it need not be.

The melting profile, including the melting temperature (T<sub>m</sub>) of the oligonucleotide/rRNA hybrids should 55 also be determined. One way to determine T<sub>m</sub> is to hybridize a <sup>32</sup>P-labelled oligonucleotide to its complementary target nucleic acid at 50°C in 0.1 M phosphate buffer, pH 6.8. The hybridization mixture is diluted and passed over a 2cm hydroxyapatite column at 50°C. The column is washed with 0.1 M phosphate buffer, 0.02% SDS to elute all unhybridized, single-stranded probes. The column temperature is

then dropped 15°C and increased in 5°C increments until all of the probe is single-stranded. At each temperature, unhybridized probe is eluted and the counts per minute (cpm) in each fraction determined. The number of cpm shown to be bound to the hydroxyapatite divided by the total cpm added to the column equals the percent hybridization of the probe to the target nucleic acid.

- 5 An alternate method for determining thermal stability of a hybrid is outlined below. An aliquot of hybrid nucleic acid is diluted into 1 ml of either 0.12M phosphate buffer, 0.2% SCS, 1mM EDTA, 1mM EGTA or an appropriate hybridization buffer. Heat this 1 ml of solution to 45 degrees C for 5 minutes and place it into a room temperature water bath to cool for 5 minutes. Assay this 1 ml of hybrid containing solution over a hydroxyapatite column, capturing the hybrid and washing away unbound probe. If a hybridization solution other than the 0.12M phosphate buffer is used, then a dilution of the hybridization solution into the 0.12M phosphate buffer cocktail will be necessary for binding. Keep taking aliquots of preformed hybrid and diluting into 1 ml of hybridization solution or into the standard 0.12M phosphate buffer solution described above while raising the heating temperature 5 degrees C at a time. Continue this until all of the hybrid is dissociated. The point where one half of the hybrid is converted to the dissociated form is considered the
- 10 Tm. The Tm for a given hybrid will vary depending on the hybridization solution being used because the thermal stability depends upon the concentration of different salts, detergents, and other solutes which effect relative hybrid stability under thermal denaturation conditions.
- 15

Because the extent and specificity of hybridization reactions such as those described herein are affected by a number of factors, manipulation of one or more of those factors will determine the exact sensitivity and specificity of a particular probe, whether perfectly complementary to its target or not. For example, the base composition of the probe may be significant because G-C base pairs exhibit greater thermal stability as compared to A-T base pairs due to additional hydrogen bonding. Thus, hybridization involving complementary nucleic acids of higher G-C content will be stable at higher temperatures.

We have discovered that the length of the target nucleic acid sequence and, accordingly, the length of the probe sequence can also be important. While it is possible for nucleic acids that are not perfectly complementary to hybridize, the longest stretch of perfectly homologous base sequence will normally primarily determine hybrid stability. While oligonucleotide probes of different lengths and base composition may be used, oligonucleotide probes preferred in this invention are between about 15 and about 50 bases in length and are at least about 75-100% homologous to the target nucleic acid. For most applications 95-100% homology to the target nucleic acid is preferred.

Ionic strength and incubation temperature should also be taken into account in constructing a probe. It is known that the rate of hybridization will increase as ionic strength of the reaction mixture increases and that the thermal stability of hybrids will increase with increasing ionic strength. In general, optimal hybridization for synthetic oligonucleotide probes of about 15-50 bases in length occurs approximately 5°C below the melting temperature for a given duplex. Incubation at temperatures below the optimum may allow mismatched base sequences to hybridize and can therefore result in reduced specificity.

As to nucleic acid concentration, it is known that the rate of hybridization is proportional to the concentration of the two interacting nucleic acid species. Thus, the presence of compounds such as dextran and dextran sulphate are thought to increase the local concentration of nucleic acid species and thereby result in an increased rate of hybridization. Other agents which will result in increased rates of hybridization are specified in U.S. Application Serial No. 627,785, entitled "Accelerated Nucleic Acid Reassociation Method", filed July 5, 1984, Continuation-In-Part thereof, Serial No. (not yet assigned), filed June 4, 1987, and U.S. Application Serial No. 816,711, entitled "Accelerated Nucleic Acid Reassociation Method", filed January 7, 1986, both of which are incorporated by reference. On the other hand, chemical reagents which disrupt hydrogen bonds such as formamide, urea, DMSO, and alcohols will increase the stringency of hybridization.

Selected oligonucleotide probes may be labelled by any of several well known methods. Useful labels include radioisotopes as well as non-radioactive reporting groups. Isotopic labels include <sup>3</sup>H, <sup>35</sup>S, <sup>32</sup>P, <sup>125</sup>I Cobalt and <sup>14</sup>C. Most methods of isotopic labelling involve the use of enzymes and include the known methods of nick translation, end labelling, second strand synthesis, and reverse transcription. When using radio-labelled probes, hybridization can be detected by autoradiography, scintillation counting, or gamma counting. The detection method selected will depend upon the hybridization conditions and the particular radioisotope used for labelling.

Non-isotopic materials can also be used for labelling, and may be introduced by the incorporation of modified nucleotides through the use of enzymes or by chemical modification of the probe, for example, by the use of non-nucleotide linker groups. Non-isotopic labels include fluorescent molecules, chemiluminescent molecules, enzymes, cofactors, enzyme substrates, haptens or other ligands. We currently prefer to use acridinium esters.

In one embodiment of the DNA/rRNA hybridization assay invention, a labelled probe and bacterial target nucleic acids are reacted in solution. rRNA may be released from bacterial cells by the sonic disruption method described in Murphy, K.A. et al., U.S. Application Serial No. 841,880, entitled "Method for Releasing RNA and DNA From Cells", filed March 20, 1986, which is incorporated herein by reference. Other known methods for disrupting cells include the use of enzymes, osmotic shock, chemical treatment, and vortexing with glass beads. Following or concurrent with the release of rRNA, labelled probe may be added in the presence of accelerating agents and incubated at the optimal hybridization temperature for a period of time necessary to achieve significant reaction. Following this incubation period, hydroxyapatite may be added to the reaction mixture to separate the probe/rRNA hybrids from the non-hybridized probe molecules. The hydroxyapatite pellet is washed, re-centrifuged and hybrids detected by means according to the label used.

Twenty-one embodiments illustrative of the claimed inventions are set forth below, in which a synthetic probe or probes complementary to a unique rRNA sequence from a target organism, or group of organisms is determined, constructed and used in a hybridization assay.

15

### DESCRIPTION OF PARTICULAR EMBODIMENTS

Mycobacterium are acid-fast, alcohol fast, aerobic, non-mobile bacilli. Their lipid content is high and their growth slow. Mycobacterium avium and Mycobacterium intracellulare are together referred to as M. avium-intracellulare because they are so difficult to differentiate. Recently, the M. avium complex, which includes M. intracellulare, was shown to be the second most commonly isolated, clinically significant Mycobacterium. Good, R.C. et al., J. Infect. Dis. 146, 829-833 (1982). More recent evidence indicates that these organisms are a common cause of opportunistic infection in patients with AIDS (acquired immune deficiency syndrome). Gill, V.J. et al., J. Clin. Microbiol. 22, 543-548 (1985). Treatment of such infections in AIDS patients is difficult because these organisms are resistant to most antituberculosis drugs. Often a combination of five drugs are used in therapy. The severity of these infections also requires rapid diagnosis which, prior to the invention herein, was not available.

Members of the Mycobacterium tuberculosis complex (Mtb) include Mycobacterium tuberculosis, Mycobacterium bovis, Mycobacterium africanum and Mycobacterium microti. The first three are pathogenic for humans while the last is an animal pathogen. These organisms produce slowly developing granulomas on the skin or they may invade internal organs. Tuberculosis of the lungs can be disseminated to other parts of the body by the circulatory system, the lymph system, or the intestinal tract. Despite advances in public health and the advent of effective chemotherapy, Mycobacterial disease, tuberculosis in particular, continues to represent a major world-wide health problem.

The classical method for detecting bacteria in a test sample involves culturing of the sample in order to expand the number of bacterial cells present into observable colony growths which can be identified and enumerated. If desired, the cultures can also be subjected to additional testing in order to determine antimicrobial susceptibility. Currently, the most widely used procedures for the detection, isolation and identification of Mycobacterium species are the acid-fast bacilli (AFB) smear (using either the Ziehl-Neelsen or fluorochrome techniques), culture methods using Lowenstein-Jensen media and Middlebrook media, and biochemical tests. The AFB relies on the high lipid content of Mycobacterium to retain dye after exposure to acid-alcohol. While the AFB smear test is relatively rapid and simple to perform it does not always detect Mycobacteria and will not differentiate between Mycobacterium avium and non-tuberculosis species, between Mycobacterium intracellulare and non-tuberculosis species, or between Mycobacterium tuberculosis-complex bacilli and non-tuberculosis species. For accurate identification of the infecting Mycobacterial species the clinician must rely on culture results which can require anywhere from 3 to 8 weeks of growth followed by extensive biochemical testing. Other tests have been developed based on the detection of metabolic products from Mycobacterium using carbon-14 labelled substrates. In particular, the Bactec (TM) instrument can detect the presence of Mycobacterium within 6 to 10 days of the time of inoculation. Gill, V.J., supra. However, the test does not distinguish Mycobacterium species. It is often important to make this determination so that particular drugs to which the organism is susceptible may be prescribed. For traditional culture methods, this requires an additional 2 to 3 weeks and for the Bactec method, an additional 6 to 10 days.

In addition, specific embodiments for Mycoplasma pneumoniae, the Mycobacterium, Liegonella, Salmonella, Chlamydia trachomatis, Campylobacter, Proteus mirabilis, Enterococcus, Enterobacter cloacae, E. coli, Pseudomonas Group I, bacteria, fungi and Neisseria gonorrhoeae are set forth in the following examples.

As indicated by the below examples, the present invention has significant advantages over each of these prior art methods not only in the enhanced accuracy, specificity and simplicity of the test, but also in greatly reducing the time to achieve a diagnosis. The invention makes possible a definitive diagnosis and initiation of effective treatment on the same day as testing.

5

### Example 1

Described below is the preparation of a single strand deoxyoligonucleotide of unique sequence and defined length which is labelled and used as a probe in a solution hybridization assay to detect the presence of rRNA from Mycobacterium avium. This unique sequence is specific for the rRNA of Mycobacterium avium and does not significantly cross-react under the hybridization conditions of this Example, with nucleic acids from any other bacterial species or respiratory infectious agent, including the closely-related Mycobacterium intracellulare. This probe is able to distinguish the two species, notwithstanding an approximate 98% rRNA approximate homology between the two species. In this Example, as well as in Examples 2 and 3, sequences for M. avium, M. tuberculosis complex, M. intracellulare and related organisms were obtained by using a specific primer to a highly conserved region in the 16S rRNA. The sequence of this primer, derived from E. coli rRNA, was 5'-GGC CGT TAC CCC ACC TAC TAG CTA AT-3'. 5 nanograms of primer was mixed with 1 microgram of each rRNA to be sequenced in the presence of 0.1M KCl and 20mM Tris-HCl pH 8.3 in a final volume of 10 microliters. The reactions were heated 10 min. at 45°C and then placed on ice. 2.5 microliters of <sup>35</sup>S dATP and 0.5 microliters of reverse transcriptase were added. The sample was aliquoted into 4 tubes, each tube containing either dideoxy A, G, T, or C. The concentrations of these nucleotides are set forth in Lane et al., supra. The samples were incubated at 40°C for 30 minutes, and were then precipitated in ethanol, centrifuged and the pellets lyophilized dry. Pellets were resuspended in 10 microliters formamide dyes (100% formamide, 0.1% bromphenol blue and 0.1% xylene cyanol), and loaded onto 80 cm 8% polyacrylamide gels. The gels were run at 2000 volts for 2-4 hours.

Thus, nucleotide sequences for the 16S rRNA of Mycobacterium avium and what were considered to be its closest phylogenetic neighbors, Mycobacterium intracellulare and Mycobacterium tuberculosis, were determined by the method of Lane, D.J. et al., Proc. Nat. Acad. Sci. USA 82:6955 (1985). In addition to determining the rRNA sequences for the organisms noted above, a spectrum of clinically significant Mycobacterium were also sequenced. These included M. fortuitum, M. scrofulaceum and M. chelonae. Selected members of several genera closely related to Mycobacterium were also sequenced, including Rhodococcus bronchialis, Corynebacterium xerosis and Nocardia asteroides.

Partial rRNA sequences from the above organisms were aligned for maximum nucleotide homology, using commercially available software from Intelligenetics, Inc., 1975 El Camino Real West, Mountain View, California 94040-2216 (IFIND Program). From this alignment, regions of sequence unique to Mycobacterium avium were determined. The probe was selected so that it was perfectly complementary to a target nucleic acidsequence and so that it had a 10% or greater mismatch with the aligned rRNA from its known closest phylogenetic neighbor. A sequence 38 bases in length was chosen. The number of mismatched bases relative to the Mycobacterium avium sequence were as follows: Mycobacterium tuberculosis (8); Mycobacterium intracellulare (5); Mycobacterium scrofulaceum (6); Mycobacterium chelonae (12); and Mycobacterium fortuitum (10).

The following cDNA sequence was characterized by the criteria of length, Tm, and sequence analysis as described at pages 7-8 above and was determined to be specific for the rRNA Mycobacterium avium:

ACCGCAAAAGCTTCCACCAGAACATGCGTCTTGAG.

This sequence is complementary to a unique segment found in the 16S rRNA of Mycobacterium avium. The size of the probe is 38 bases. The probe has a Tm of 74°C and sequence analysis by the method of Maxam & Gilbert (1980), supra, confirmed that the probe was correctly synthesized. The probe is capable of hybridizing to rRNA of M. avium in the region corresponding to bases 185-225 of E. coli 16S rRNA.

To demonstrate the reactivity of this sequence for Mycobacterium avium, it was tested as a probe in hybridization reactions under the following conditions. <sup>32</sup>P-end-labeled oligonucleotide probes were mixed with 1 microgram ( $7 \times 10^{-13}$  moles) of purified rRNA from Mycobacterium avium and reacted in 0.12 M PB hybridization buffer (equimolar amounts of Na<sub>2</sub>HPO<sub>4</sub> and NaH<sub>2</sub>PO<sub>4</sub>), 1 mM EDTA and 0.02% SDS (sodium dodecyl sulfate) at 65°C for 60 minutes in a final volume of 50 microliters. In separate tubes the probe was

mixed with the hybridization buffer both with and without target present. Following separation on hydroxyapatite as outlined in the patent applications identified at page 2, supra, the hybrids were quantitated by scintillation counting. These results are presented in Table 1, showing that the probe has a high extent of reaction to homologous target and very little non-specific binding to the hydroxyapatite.

5

TABLE 1  
HYBRIDIZATION OF THE M. AVIUM PROBE  
TO HOMOLOGOUS TARGET rRNA\*

	<u>plus rRNA</u>	<u>minus rRNA</u>
M. avium probe	85-95%	0.5%
* %Hybridization = <u>cpm bound to hydroxyapatite</u> <u>total cpm added to reaction</u>		

20 Specificity of the probe for M. avium was tested by mixing the  $^{32}\text{P}$  labeled probe with rRNA released from cells of 29 other species of mycobacteria by the sonic disruption techniques described in Murphy et al., U.S. Application Serial No. 841,860.  $1 \times 10^8$  cells were suspended in 0.1 ml 5% SDS and sonicated for 10 minutes at 50-60°C. 1.0 ml of hybridization buffer (45% sodium dodecyl sulfate, 40 mM phosphate buffer pH 8.8 and 1 mM EDTA) was added and the mixture incubated for 60 minutes at 72°C.  
25 Following incubation, 4.0 ml of hydroxyapatite solution (0.14M sodium phosphate buffer, pH 8.8, 0.02% SDS and 1.0 gram hydroxyapatite per 50 mls solution) was added and incubated for 5 minutes at 72°C. The sample was centrifuged and the supernatant removed. 4.0 ml wash solution (0.14 M sodium phosphate pH 8.8) was added and sample was vortexed, centrifuged and the supernatant removed. The radioactivity bound to the hydroxyapatite was determined by scintillation counting. The results are shown in Table 2 and indicate that the probe is specific for Mycobacterium avium and does not react with any other mycobacterial species, including Mycobacterium intracellulare.

35

40

45

50

55

TABLE 2

5 HYBRIDIZATION OF THE *M. AVIUM* PROBE TO MYCOBACTERIAL SPECIES

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
	<i>Mycobacterium africanum</i>	25420	1.0
10	<i>M. asiaticum</i>	25276	1.2
	<i>M. avium</i>	25291	87.6
	<i>M. bovis</i>	19210	1.2
	<i>M. bovis (BCG)</i>	19015	1.0
	<i>M. chelonae</i>	14472	0.9
15	<i>M. flavescens</i>	14474	0.9
	<i>M. fortuitum</i>	6841	1.0
	<i>M. gastri</i>	15754	1.2
	<i>M. gordoneae</i>	14470	1.2
	<i>M. haemophilum</i>	29548	1.3
	<i>M. intracallulare</i>	13950	1.5
20	<i>M. kansasii</i>	12478	1.2
	<i>M. malmoense</i>	29571	1.2
	<i>M. marinum</i>	827	1.2
	<i>M. nonchromogenicum</i>	1930	1.1
	<i>M. phlei</i>	11758	1.3
25	<i>M. scrofulaceum</i>	19981	1.2
	<i>M. shimoidei</i>	27962	2.3
	<i>M. simiae</i>	25275	1.2
	<i>M. smegmatis</i>	el4468	1.0
	<i>M. szulgai</i>	23069	1.0
	<i>M. terrae</i>	15755	1.2
30	<i>M. thermoresistibile</i>	19527	1.3
	<i>M. triviale</i>	23292	1.2
	<i>M. tuberculosis (avirulent)</i>	25177	1.4
	<i>M. tuberculosis (virulent)</i>	27294	1.1
	<i>M. ulcerans</i>	19423	1.4
	<i>M. vaccae</i>	15483	1.2
35	<i>M. xenopi</i>	19971	1.5

As shown in Table 3 the probe also did not react with the rRNA from any of the respiratory pathogens which were also tested by the method just described. Nor did the probe react with any other closely related or phylogenetically more diverse species of bacteria also tested by that method (Table 4).

40

45

50

55

TABLE 3

HYBRIDIZATION OF M. AVIUM PROBE TO  
RESPIRATORY PATHOGENS

	<u>Organism</u>	<u>ATCC#</u>	<u># Probe Bound</u>
10	Corynabacterium xerosis	373	0.7
	Fusobacterium nucleatum	25586	1.3
	Haemophilus influenzae	19418	1.3
	Klebsiella pneumoniae	23357	1.8
15	Legionella pneumophila	33152	0.0
	Mycoplasma pneumoniae	15531	3.0
	Neisseria meningitidis	13090	0.0
	Pseudomonas aeruginosa	25330	0.0
	Propionibacterium acnes	6919	1.1
	Streptococcus pneumoniae	6306	0.0
20	Staphylococcus aureus	25923	1.5

TABLE 4

HYBRIDIZATION OF THE M. AVIUM PROBE TO A PHYLOGENETIC  
CROSS SECTION OF BACTERIAL SPECIES

	<u>Organism</u>	<u>ATCC#</u>	<u># Probe Bound</u>
30	Acinetobacter calcoaceticus	33604	0.0
	Branhamella catarrhalis	25238	0.6
	Bacillus subtilis	6051	0.9
	Bacteroides fragilis	23745	1.0
	Campylobacter jejuni	33560	0.4
35	Chromobacterium violaceum	29094	1.7
	Clostridium perfringens	13124	2.1
	Deinococcus radiodurans	35073	0.8
	Derxia gummosa	15994	0.3
	Enterobacter aerogenes	13048	0.6
40	Escherichia coli	11775	0.3
	Mycobacterium gordoneae	14470	1.9
	Mycoplasma hominis	14027	3.3
	Proteus mirabilis	29906	0.0
	Pseudomonas cepacia	11762	1.0
	Rahnella aquatilis	33071	2.1
45	Rhodospirillum rubrum	11170	0.6
	Streptococcus mitis	9811	0.9
	Vibrio parahaemolyticus	17802	1.2
	Versinia enterocolitica	9610	0.4

Example 2

After the alignment described in Example 1, the following sequence was characterized by the aforementioned criteria of length, Tm and sequence analysis and was determined to be specific for *Mycobacterium intracellulare*:

ACCGCAAAAGCTTCCACCTAAAGACATGCGCCTAAAG

The sequence is complementary to a unique segment found in the 16S rRNA of *Mycobacterium intracellulare*. The size of the probe was 38 bases. The probe has a Tm of 75°C and sequence analysis

6 TABLE 3

HYBRIDIZATION OF M. AVIUM PROBE TO  
RESPIRATORY PATHOGENS

	<u>Organism</u>	<u>ATCC†</u>	<u>% Probe Bound</u>
10	Corynebacterium xerosis	373	0.7
	Fusobacterium nucleatum	25586	1.3
	Haemophilus influenzae	19418	1.3
	Klebsiella pneumoniae	23357	1.8
15	Legionella pneumophila	33152	0.0
	Mycoplasma pneumoniae	15531	3.0
	Neisseria meningitidis	13090	0.0
	Pseudomonas aeruginosa	25330	0.0
	Propionibacterium acnes	6919	1.1
	Streptococcus pneumoniae	6306	0.0
20	Staphylococcus aureus	25923	1.5

25 TABLE 4

HYBRIDIZATION OF THE M. AVIUM PROBE TO A PHYLOGENETIC  
CROSS SECTION OF BACTERIAL SPECIES

	<u>Organism</u>	<u>ATCC†</u>	<u>% Probe Bound</u>
30	Acinetobacter calcoaceticus	33604	0.0
	Branhamella catarrhalis	25238	0.6
	Bacillus subtilis	6051	0.9
	Bacteroides fragilis	23745	1.0
	Campylobacter jejuni	33560	0.4
35	Chromobacterium violaceum	29094	1.7
	Clostridium perfringens	13124	2.1
	Deinococcus radiodurans	35073	0.8
	Dexia gummosa	15994	0.3
	Enterobacter aerogenes	13048	0.6
	Escherichia coli	11775	0.3
40	Mycobacterium gordoneae	14470	1.9
	Mycoplasma hominis	14027	3.3
	Proteus mirabilis	29906	0.0
	Pseudomonas cepacia	11762	1.0
	Rahnella aquatilis	33071	2.1
45	Rhodospirillum rubrum	11170	0.6
	Streptococcus mitis	9811	0.9
	Vibrio parahaemolyticus	17802	1.2
	Yersinia enterocolitica	9610	0.4

50

Example 2

After the alignment described in Example 1, the following sequence was characterized by the aforementioned criteria of length, Tm and sequence analysis and was determined to be specific for *Mycobacterium intracellulare*:

ACCGCAAAAGCTTCCACCTAAAGACATGCGCCTAAAG

The sequence is complementary to a unique segment found in the 16S rRNA of *Mycobacterium intracellulare*. The size of the probe was 38 bases. The probe has a Tm of 75°C and sequence analysis

confirmed that the probe was correctly synthesized. The probe hybridizes to RNA of M. intracellulare in the region corresponding to bases 185-225 of E. coli 16S rRNA.

To demonstrate the reactivity of this sequence for the Mycobacterium intracellulare, the probe was tested in hybridization reactions under the following conditions. <sup>32</sup>P-end-labelled oligonucleotide probe was mixed with 1 microgram ( $7 \times 10^{-3}$  moles) of purified rRNA from Mycobacterium intracellulare and reacted in 0.12 M PB (equimolar amounts of Na<sub>2</sub>HPO<sub>4</sub> and NaH<sub>2</sub>PO<sub>4</sub>), 1 mM EDTA and 0.2% SDS (sodium dodecyl sulfate) at 65°C for 60 minutes in a final volume of 50 microliters. In separate tubes the probe was mixed with the hybridization buffer with and without target Mycobacterium intracellulare rRNA present. Following separation on hydroxyapatite as outlined previously the hybrids were quantitated by scintillation counting. These results are shown in Table 5.

TABLE 5

HYBRIDIZATION OF THE M. INTRACELLULARE PROBE  
TO HOMOLOGOUS TARGET rRNA\*

	<u>plus rRNA</u>	<u>minus rRNA</u>
20 <u>M. intracellulare</u> probe	85-95%	0.5%

\* % Hybridization = cpm bound to hydroxyapatite  
total cpm added to reaction

25      These data shows that the probe has a high extent of reaction to its homologous target and very little non-specific binding to the hydroxyapatite.

Specificity of the Mycobacterium intracellulare probe was tested by mixing the <sup>32</sup>P labelled probe with rRNA released from cells from 29 other species of mycobacteria by sonic disruption techniques described in Murphy et al. U.S. Patent Application No. 841,880. All hybridization assays were carried out as described in Example 1. Table 6 indicated that the probe is specific for Mycobacterium intracellulare and does not react with any other mycobacterial species, including Mycobacterium avium. These results are impressive in view of the 98% rRNA homology to M. avium; 98% homology to M. kansasii; 98% homology to M. asiaticum; and 97% homology to M. tuberculosis.

TABLE 6

HYBRIDIZATION OF THE M. INTRACELLULARE PROBE  
TO MYCOBACTERIAL SPECIES

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
50 <u>Mycobacterium africanum</u>	25420	0.9	
	25276	1.1	
	25291	1.3	
	19210	1.1	

	M. bovis (BCG)	19015	1.2
5	M. chelonae	14472	1.0
	M. favescens	14474	1.2
	M. fortuitum	6841	1.3
	M. gastri	15754	1.3
	M. gordonae	14470	1.3
	M. haemophilum	29548	0.9
	M. intracellulare	13950	78.8
	M. kansasii	12479	1.1
10	M. Malmense	29571	1.0
	M. marinum	827	0.9
	M. nonchromogenicum	1930	1.0
	M. phlei	11758	1.1
	M. scrofulaceum	19981	1.0
15	M. shimoidei	27962	1.3
	M. simiae	25275	1.1
	M. smegmatis	214468	1.3
	M. szulgai	23069	1.0
	M. terrae	15755	1.4
	M. thermoresistibile	19527	1.6
20	M. triviale	23292	1.3
	M. tuberculosis (avirulent)	25177	1.2
	M. tuberculosis (virulent)	27294	1.2
	M. ulcerans	19423	1.1
	M. vaccae	15483	1.0
25	M. xenopi	19971	1.2

As shown in Table 7 the probe did not react with the rRNA from any of the respiratory pathogens tested in the hybridization assay. Nor did the probe react with any other closely related or phylogenetically more diverse species of bacteria that were tested (Table 8).

30

35

TABLE 7

HYBRIDIZATION OF THE M. INTRACELLULARE PROBE  
TO RESPIRATORY PATHOGENS

	<u>Organism</u>	<u>ATCC#</u>	<u># Probe Bound</u>
	Corynebacterium xerosis	373	2.2
45	Fusobacterium nucleatum	25586	1.5
	Haemophilus influenzae	19418	1.3
	Klebsiella pneumoniae	23357	1.2
	Legionella pneumophila	33152	1.2
	Mycoplasma pneumoniae	15531	3.2
	Neisseria meningitidis	13090	1.1
50	Pseudomonas aeruginosa	25330	1.0
	Propionibacterium acnes	6919	2.9
	Streptococcus pneumoniae	6306	1.6
	Staphylococcus aureus	25923	1.3

55

TABLE 8

5

HYBRIDIZATION OF THE M. INTRACELLULARE PROBE  
TO A PHYLOGENETIC CROSS SECTION OF BACTERIAL SPECIES

	<u>Organism</u>	<u>ATTC#</u>	<u>% Probe</u>
10	<i>Acinetobacter calcoaceticus</i>	33604	1.5
	<i>Branhamella catarrhalis</i>	25238	1.8
	<i>Bacillus subtilis</i>	6051	1.7
	<i>Bacteroides fragilis</i>	23745	1.9
	<i>Campylobacter jejuni</i>	33560	1.9
15	<i>Chromobacterium Viciaceum</i>	29094	1.4
	<i>Clostridium perfringens</i>	13124	2.1
	<i>Deinococcus radiodurans</i>	35073	2.1
	<i>Dexia gummosa</i>	15994	1.6
	<i>Enterobacter aerogenes</i>	13048	1.3
20	<i>Escharichia coli</i>	11775	1.2
	<i>Mycobacterium gordonaiae</i>	14470	2.3
	<i>Mycoplasma hominis</i>	14027	2.6
	<i>Proteus mirabilis</i>	29906	1.2
	<i>Pseudomonas cepacia</i>	11762	1.7
	<i>Rahnella aquatilis</i>	33071	1.5
25	<i>Rhodospirillum rubrum</i>	11170	1.4
	<i>Strptococcus mitis</i>	9811	1.4
	<i>Vibrio parahaemolyticus</i>	17802	2.5
	<i>Yersinia enterocolitica</i>	9610	1.1

30

Example 3

After the alignment described in Example 1, the following sequence was characterized by the aforementioned three criteria of size, sequence and Tm, and was determined to be specific to the Mtb complex of organisms, *Mycobacterium tuberculosis*, *Mycobacterium africanum*, *Mycobacterium bovis*, and *Mycobacterium microti*:

1. TAAAGCGCTTCCACCACAAGACATGCATCCCGTG.

The sequence is complementary to a unique segment found in the 16S rRNA of the Mtb-complex bacteria. The size of the probe is 35 bases. The probe has a Tm of 72°C and sequence analysis confirmed that the probe was correctly synthesized. It is capable of hybridizing in the region corresponding to bases 185-225 of *E. coli* 16S rRNA.

To demonstrate the reactivity of this sequence for the Mtb complex the probe was tested in hybridization reactions under the following conditions. <sup>32</sup>P-end-labelled oligonucleotide probe was mixed with 1 microgram ( $7 \times 10^{-13}$  moles) of purified rRNA from *Mycobacterium tuberculosis* and reacted in 0.12 M PB hybridization buffer (equimolar amounts of Na<sub>2</sub>HPO<sub>4</sub> and NaH<sub>2</sub>PO<sub>4</sub>), 1 mM EDTA and 0.2 SDS (sodium dodecyl sulfate) at 65°C for 60 minutes in a final volume of 50 microliters. In separate tubes the probe was mixed with the hybridization buffer with and without target rRNA from *Mycobacterium tuberculosis* present. Following separation on hydroxyapatite as outlined previously the hybrids were quantitated by scintillation counting. The results are shown in Table 9.

50

55

TABLE 9

5 HYBRIDIZATION OF Mtb-COMPLEX 16S rRNA DNA PROBE  
TO HOMOLOGOUS TARGET rRNA\*

	<u>plus rRNA</u>	<u>minus rRNA</u>
10 Mtb complex probe	85-95%	0.5%
* % Hybridization = <u>cpm bound to hydroxyapatite</u> <u>total cpm added to reaction</u>		

15 This data shows that the probe has a high extent of reaction to homologous target and very little non-specific binding to the hydroxyapatite.

Specificity of the probe for the Mtb complex was tested by mixing the <sup>32</sup>P labelled probe with rRNA released from cells of the 4 Mtb complex bacilli and of 25 other mycobacterial species by sonic disruption techniques described in Murphy et al., U.S. Patent Application No. 841,880. All hybridization assays were 20 carried out as described in Example 1. Table 10 indicates that the probe is specific for organisms within the Mtb complex and does not react with any other mycobacterial species.

TABLE 10

25 HYBRIDIZATION OF Mtb-COMPLEX 16S rRNA DNA PROBE  
TO MYCOBACTERIAL SPECIES

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
30	Mycobacterium africanum	25420	68.1
	M. asiaticum	25276	3.4
	M. avium	25291	0.9
	M. bovis	19210	63.1
	M. chelonae	14472	1.1
35	M. flavescens	14474	0.9
	M. fortuitum	6841	1.1
	M. gastri	15754	0.8
	M. gordoneae	14470	1.1
	M. haemophilum	29548	0.8
40	M. intracallulare	13950	1.1
	M. kansasii	12479	1.3
	M. malmoense	29571	0.9
	M. marinum	827	1.1
	M. nonchromogenicum	1930	1.1
	M. phlei	11758	1.3
45	M. scrofulaceum	19981	1.1
	M. shimoidei	27962	1.0
	M. simiae	25275	1.2
	M. smegmatis	e14468	0.9
	M. szulgai	23069	1.1
	M. tarrae	15755	1.0
50	M. thermoresistibile	19527	1.0
	M. triviale	23292	1.2
	M. tuberculosis (avirulent)	25177	66.2
	M. tuberculosis (virulent)	27294	62.4
	M. ulcerans	19423	0.9
55	M. vaccae	15483	0.8
	M. xenopi	19971	2.6

As shown in Table 11 the probe did not react with the rRNA from any of the respiratory pathogens tested in the hybridization assay. Nor did the probe react with any other closely related or phylogenetically more diverse species of bacteria that were tested (Table 12).

6

TABLE 11

HYBRIDIZATION OF Mtb-COMPLEX 16S rRNA DNA PROBE  
TO RESPIRATORY PATHOGENS

10

<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
Corynebacterium xerosis	373	1.3
Fusobacterium nucleatum	25586	1.0
Haemophilus influenzae	19418	1.6
Klebsiella pneumoniae	23357	1.2
Legionella pneumophila	33152	1.4
Mycoplasma pneumoniae	15531	1.1
Neisseria meningitidis	13090	1.0
Pseudomonas aeruginosa	25330	1.7
Propionibacterium acnes	6919	1.2
Streptococcus pneumoniae	25923	0.9

25

TABLE 12

HYBRIDIZATION OF THE Mtb-COMPLEX 16S rRNA DNA PROBE  
TO A PHYLOGENETIC CROSS SECTION OF BACTERIAL SPECIES

30

<u>Organism</u>	<u>ATCC#</u>	<u>% Probe</u>
Acinetobacter calcoaceticus	33604	1.3
Branhamella catarrhalis	25238	1.5
Bacillus subtilis	6051	1.3
Bacteroides fragilis	23745	1.3
Campylobacter jejuni	33560	1.1
Chromobacterium violaceum	29094	1.0
Clostridium perfringens	13124	1.2
Dainococcus radiodurans	35073	1.0
Derxia gummosa	15994	1.0
Enterobacter aerogenes	13048	1.0
Escherichia coli	11775	1.0
Mycobacterium gordonaiae	14470	1.3
Mycoplasma hominis	14027	0.5
Proteus mirabilis	29906	1.0
Pseudomonas cepacia	11762	2.6
Rahnella aquatilis	33071	1.9
Rhodospirillum rubrum	11170	1.0
Streptococcus mitis	9811	1.1
Vibrio parahaemolyticus	17802	0.9
Yersinia enterocolitica	9610	1.1

60

Two derivatives of the probe of Example 3 (numbered 2-3 below) were made and tested:

2. CCGCTAAAGCGCTTCCACCAAGACATGCATCCG
3. ACACCGCTAAAGCGCTTCCACCAAGACATGCATC.

55 All three probes have similar Tms (72°; 73.5°; and 72.3°, respectively) and similar hybridization characteristics.

Hybridization to Mycobacterium tuberculosis complex organisms was 68-75% and non-specific hybridization to hydroxyapatite was less than 2%. Results of hybridization assay tests for these derivatives

follow.

5

TABLE 13

**HYBRIDIZATION OF PROBE OF EXAMPLES 3 AND 2  
DERIVATIVES THEREOF  
TO MYCOBACTERIAL SPECIES**

10

<u>Organism</u>	<u>ATCC#</u>	<u>Example</u>		
		<u>% Probe 1 Bound</u>	<u>% Probe 2 Bound</u>	<u>% Probe 3 Bound</u>
<i>Mycobacterium africanum</i>	25420	68.1	69.4	70.6
<i>M. asiaticum</i>	25274	3.4	5.3	1.8
<i>M. avium</i>	25291	0.9	1.6	1.4
<i>M. bovis</i>	19210	63.1	75.3	74
<i>M. chalonae</i>	14472	1.1	1.5	1.6
<i>M. flavescens</i>	14474	0.9	2.7	1.4
<i>M. fortuitum</i>	6841	1.1	3.6	1.5
<i>M. gastri</i>	15754	0.8	3.6	1.7
<i>M. gordonaee</i>	14470	1.1	1.6	1.4
<i>M. haemophilum</i>	29548	0.8	3.2	1.7
<i>M. intracellulare</i>	13950	1.1	1.6	1.4
<i>M. kansasii</i>	12478	1.3	2.1	2.0
<i>M. malmoense</i>	29571	0.9	2.8	1.5
<i>M. marinum</i>	827	1.1	2.1	1.5
<i>M. nonchromogenicum</i>	1930	1.1	3.0	1.5
<i>M. phlei</i>	11758	1.3	1.3	1.1
<i>M. scrofulaceum</i>	19981	1.1	3.4	1.6
<i>M. shimoidei</i>	27962	1.0	2.7	1.6
<i>M. simiae</i>	25275	1.2	2.9	1.8
<i>M. smegmatis</i>	el4468	0.9	1.5	1.2
<i>M. szulgai</i>	23069	1.1	3.6	1.1
<i>M. terrae</i>	15755	1.0	3.7	2.0
<i>M. thermoresistibile</i>	19527	1.0	1.6	1.3
<i>M. triviale</i>	23292	1.2	1.6	2.0
<i>M. tuberculosis</i> (avirulent)	25177	66.2	75	68
<i>M. tuberculosis</i> (virulent)	27294	62.4	74	75
<i>M. ulcerans</i>	19423	0.9	1.7	3.0
<i>M. vaccae</i>	15483	0.8	1.4	1.2
<i>M. xenopi</i>	19971	2.6	1.4	1.2

45

Example 4

The probe specific for the 23S rRNA of the *M. tuberculosis* complex was obtained by using a primer which was complementary to a highly conserved region of 23S rRNA. The sequence of this primer, derived from *E. coli* rRNA, was 5'-AGG AAC CCT TGG GCT TTC GG-3'. Five nanograms of this primer was mixed with 1 microgram of rRNA from *M. tuberculosis* and other closely related *Mycobacterium* and the procedure as described for Examples 1, 2 and 3 was followed. After alignment as described in Example 1, the following sequence was determined to be specific to the Mtb complex of organisms, *Mycobacterium tuberculosis*, *Mycobacterium africanum*, *Mycobacterium bovis*, and *Mycobacterium microti*:

56      TGCCCTACCCACACCCCACCACAAAGGTGATGT.

The sequence is complementary to a unique segment found in the 23S rRNA of the Mtb-complex bacteria. The oligonucleotide probe was characterized as previously described by the criteria of length, Tm and sequence analysis. The size of the probe is 31 bases. The probe has a Tm of 72.5°C and sequence

analysis confirmed that the probe was correctly synthesized. It is capable of hybridizing in the region corresponding to bases 1155-1180 of *E. coli* 23S rRNA.

To demonstrate the reactivity of this sequence for the Mtb complex the probe was tested in hybridization reactions under the following conditions.  $^{32}\text{P}$ -end-labelled oligonucleotide probes were mixed with 1 microgram ( $7 \times 10^{-13}$  moles) of purified rRNA from *Mycobacterium tuberculosis* and reacted in 0.12 M PB hybridization buffer (equimolar amounts of  $\text{Na}_2\text{HPO}_4$  and  $\text{NaH}_2\text{PO}_4$ ), 1 mM EDTA and 0.2 SDS (sodium dodecyl sulfate) at 65°C for 60 minutes in a final volume of 50 microliters. In separate tubes the probe was mixed with the hybridization buffer with and without target rRNA from *Mycobacterium tuberculosis* present. Following separation on hydroxyapatite as outlined previously the hybrids were quantitated by scintillation counting. The results are shown in Table 14.

TABLE 14

18 HYBRIDIZATION OF THE Mtb-COMPLEX  
 20 23S rRNA DNA PROBE TO HOMOLOGOUS TARGET rRNA

	<u>plus rRNA</u>	<u>minus rRNA</u>
20 Mtb complex 23S probe	94%	1.2%

These data show that the probe has a high extent of reaction to homologous target and very little non-specific binding to the hydroxyapatite.

25 Specificity of the probe for the Mtb complex was tested by mixing the  $^{32}\text{P}$  labelled probe with rRNA released from cells of the four Mtb complex bacilli and of 25 other mycobacterial species by sonic disruption techniques described in Murphy et al., U.S. Patent Application No. 841,860. All hybridization assays were carried out as described in Example 1. Table 14 indicates that the probe is specific for 30 organisms within the Mtb complex and does not react with any other mycobacterial species.

35

40

45

50

55

5 TABLE 15

HYBRIDIZATION OF Mtb-COMPLEX 23S rRNA DNA PROBE  
TO MYCOBACTERIAL SPECIES

	<u>Organism</u>	<u>ATCC#</u>	<u># Probe Bound</u>
10	<i>Mycobacterium africanum</i>	25420	33.6
	<i>M. asiaticum</i>	25276	1.2
	<i>M. avium</i>	25291	1.0
15	<i>M. bovis</i>	19210	32.0
	<i>M. chelonae</i>	14472	1.2
	<i>M. flavescens</i>	14474	1.2
	<i>M. fortuitum</i>	6841	1.3
	<i>M. gastri</i>	15754	1.1
20	<i>M. gordoneae</i>	14470	1.2
	<i>M. haemophilum</i>	29548	1.2
	<i>M. intracellulare</i>	13950	1.1
	<i>M. kansasii</i>	12479	1.3
25	<i>M. malmoense</i>	29571	1.3
	<i>M. marinum</i>	827	1.2
	<i>M. nonchromogenicum</i>	1930	1.0
	<i>M. phlei</i>	11758	1.0
30	<i>M. scrofulaceum</i>	19981	1.1
	<i>M. shimoidei</i>	27962	1.2
	<i>M. simiae</i>	25275	1.3
	<i>M. smegmatis</i>	el4468	1.1
	<i>M. szulgai</i>	23069	1.1
35	<i>M. terrae</i>	15755	1.0
	<i>M. thermoresistibile</i>	19527	1.2
	<i>M. triviale</i>	23292	1.0
	<i>M. tuberculosis (avirulent)</i>	25177	33.7
	<i>M. tuberculosis (virulent)</i>	27294	38.1
	<i>M. ulcerans</i>	19423	1.3
40	<i>M. vaccae</i>	15483	1.0
	<i>M. xenopi</i>	19971	1.3

40 Example 5

Three additional *Mycobacterium tuberculosis* complex probes, Examples 5-7 herein, were identified using two unique primers complementary to 23S rRNA. The first sequence is:

CCATCACCAACCCTCTCCGGAGAGGAAAAGG.

45 The sequence of this Example 5 was obtained using a 23S primer with the sequence 5'-GGC CAT TAG ATC ACT CC-3'. It was characterized and shown to be specific for the *Mycobacterium tuberculosis* complex of organisms including *Mycobacterium tuberculosis*, *Mycobacterium africanum* and *Mycobacterium bovis*. This sequence, from 23S rRNA, is 31 bases in length and has a Tm of 72°C. This probe is capable of hybridizing to RNA of the aforementioned organisms in the region corresponding to bases 540-575 of *E. coli* 23S rRNA.

50 To demonstrate the reactivity and specificity of this probe for *Mycobacterium tuberculosis* complex, it was tested as a probe in hybridization reactions under the following conditions. <sup>32</sup>P-end-labeled oligonucleotide probe was mixed with rRNA released from cells of 30 species of mycobacteria by the sonic disruption techniques described in Murphy et al., U.S. Patent Application Serial No. 841,860. 3 × 10<sup>7</sup> cells were suspended in 0.1 ml 5% SDS and sonicated for 15 minutes at 50-60°C. One ml of hybridization buffer

55 (45% dodecyl sulfate, 40 mM phosphate buffer pH 6.8, 1 mM EDTA, 1 mM EGTA) was added and the mixture incubated at 72°C for 2 hours. Following incubation, 4 ml of 2% (w/v) hydroxyapatite, 0.12M sodium phosphate buffer pH 8.8, 0.02% SDS, 0.02% sodium azide was added and incubated at 72°C for 5 minutes. The sample was centrifuged and the supernatant removed. Four ml wash solution (0.12M sodium

phosphate buffer pH8.8, 0.02% SDS, 0.02% sodium azide) was added and the sample was vortexed, centrifuged and the supernatant removed. The radioactivity bound to the hydroxyapatite was determined by scintillation counting. The results are shown in Table 16 and indicate that the probe is specific for the Mycobacterium tuberculosis complex of organisms.

5

TABLE 16

10 HYBRIDIZATION OF THE M. TUBERCULOSIS COMPLEX  
PROBE OF EXAMPLE 5 TO MYCOBACTERIAL SPECIES

	<u>Organism</u>	<u>ATCC #</u>	<u>% Probe Bound</u>
	<i>Mycobacterium africanum</i>	25420	18.0
15	<i>M. asiaticum</i>	25274	2.6
	<i>M. avium</i>	25291	3.4
	<i>M. bovis</i>	19210	21.7
	<i>M. bovis (BCG)</i>	35734	35.3
	<i>M. chelonae</i>	14472	3.8
20	<i>M. flavescens</i>	14474	2.3
	<i>M. fortuitum</i>	6841	1.8
	<i>M. gastri</i>	15754	2.2
	<i>M. gordonae</i>	14470	2.8
	<i>M. haemophilum</i>	29548	2.8
	<i>M. intracellulare</i>	13950	2.1
25	<i>M. kansasii</i>	12478	1.6
	<i>M. malmoense</i>	29571	2.3
	<i>M. marinum</i>	827	2.1
	<i>M. nonchromogenicum</i>	1930	2.3
	<i>M. phlei</i>	11758	2.1
30	<i>M. scrofulaceum</i>	19981	2.2
	<i>M. shimoidei</i>	27962	1.9
	<i>M. simiae</i>	25275	2.2
	<i>M. smegmatis</i>	e14468	2.0
	<i>M. szulgai</i>	23069	2.2
	<i>M. terrae</i>	15755	2.2
35	<i>M. thermoresistible</i>	19527	2.2
	<i>M. triviale</i>	23292	2.0
	<i>M. tuberculosis (avirulent)</i>	25177	26.4
	<i>M. tuberculosis (virulent)</i>	27294	36.6
	<i>M. ulcerans</i>	19423	2.5
	<i>M. vaccae</i>	15483	2.4
40	<i>M. xenopi</i>	19971	2.8

45 Table 16 shows that the probe also did not cross react with RNA from any of the closely related organisms tested by the method just described.

50

55

TABLE 17

5

HYBRIDIZATION OF THE *M. TUBERCULOSIS* COMPLEX PROBE  
OF EXAMPLE 5 TO PHYLOGENETICALLY CLOSELY RELATED ORGANISMS

	<u>Organism</u>	<u>ATCC#</u>	<u># Probe Bound</u>
10	<i>Actinomadura madurae</i>	19425	2.1
	<i>Actinoplanes italicus</i>	10049	3.1
	<i>Arthrobacter oxidans</i>	14358	2.1
	<i>Brevibacterium linens</i>	e9172	1.9
	<i>Corynebacterium xerosis</i>	373	2.2
15	<i>Dermatophilus congolensis</i>	14367	2.2
	<i>Microbacterium lacticum</i>	8180	2.1
	<i>Nocardia asteroides</i>	19247	2.0
	<i>Nocardia brasiliensis</i>	19296	2.2
	<i>Nocardia otitidis-caviae</i>	14629	2.0
20	<i>Nocardiopsis dassonvillei</i>	23218	4.0
	<i>Oerskovia turbata</i>	33225	2.2
	<i>Oerskovia xanthineolytica</i>	27402	2.0
	<i>Rhodococcus aichiensis</i>	33611	1.9
	<i>Rhodococcus aurantiacus</i>	25938	2.0
	<i>Rhodococcus bronchialis</i>	25592	2.1
25	<i>Rhodococcus chubuensis</i>	33609	2.3
	<i>Rhodococcus equi</i>	6939	2.4
	<i>Rhodococcus obuensis</i>	33610	2.2
	<i>Rhodococcus sputi</i>	29627	2.3

30

Example 6

The second *Mycobacterium tuberculosis* complex probe was obtained using a 23S primer with the sequence 5' CCT GAT TGC CGT CCA GGT TGA GGG AAC CTT TGG G-3'. Its sequence is:

35           CTGTCCTAAACCGATTCAAGGGTTCGAGGTTAGATGC

This sequence, from 23S rRNA, is 38 bases in length and has a Tm of 75°C. It hybridizes in the region corresponding to bases 2195-2235 of *E. coli* 23S rRNA.

Like the complex probe in Example 5, this sequence was characterized and shown to be specific for the *Mycobacterium tuberculosis* complex of organisms including *Mycobacterium tuberculosis*, *Mycobacterium africanum* and *Mycobacterium bovis*.

To demonstrate the reactivity and specificity of the probe of this Example 6 to *Mycobacterium tuberculosis* complex, it was tested as a probe in hybridization reactions under the following conditions described for the probe in Example 5. The results are shown in Table 18 and indicate that the probe is specific for the *Mycobacterium tuberculosis* complex of organisms with the exception of *Mycobacterium thermoresistibile*, a rare isolate which is not a human pathogen.

50

55

TABLE 18

5 HYBRIDIZATION OF THE M. TUBERCULOSIS COMPLEX  
 PROBE OF EXAMPLE 6 TO MYCOBACTERIAL SPECIES

	<u>Organism</u>	<u>ATCC #</u>	<u># Probe Bound</u>
10	Mycobacterium africanum	25420	56.0
	M. asiaticum	25274	3.1
	M. avium	25291	2.6
	M. bovis	19210	48.0
	M. bovis (BCG)	35734	63.0
	M. chelonae	14472	2.8
15	M. flavescens	14474	2.8
	M. fortuitum	6841	3.0
	M. gastri	15754	3.2
	M. gordonae	14470	3.0
	M. haemophilum	29548	3.0
20	M. intracellulare	13950	3.6
	M. kansasii	12478	3.9
	M. malmoense	29571	2.9
	M. marinum	827	2.9
	M. nonchromogenicum	1930	4.8
	M. phlei	11758	2.9
25	M. scrofulaceum	19981	2.6
	M. shimoidei	27962	3.6
	M. simiae	25275	3.3
	M. smegmatis	e14468	3.0
	M. szulgai	23069	2.8
	M. terrae	15755	2.8
30	M. thermoresistibile	19527	11.7
	M. triviale	23292	3.2
	M. tuberculosis (avirulent)	25177	65.0
	M. tuberculosis (virulent)	27294	53.0
	M. ulcerans	19423	2.5
35	M. vaccae	15483	2.8
	M. xenopi	19971	3.3

Table 19 shows that the probe also did not cross react with RNA from any of the phylogenetically closely related organisms tested by the method just described.

45

50

55

TABLE 18

5 HYBRIDIZATION OF THE *M. TUBERCULOSIS* COMPLEX  
 PROBE OF EXAMPLE 6 TO MYCOBACTERIAL SPECIES

	<u>Organism</u>	<u>ATCC #</u>	<u>% Probe Bound</u>
10	<i>Mycobacterium africanum</i>	25420	56.0
	<i>M. asiaticum</i>	25274	3.1
	<i>M. avium</i>	25291	2.6
	<i>M. bovis</i>	19210	48.0
	<i>M. bovis (BCG)</i>	35734	63.0
	<i>M. chelonae</i>	14472	2.8
15	<i>M. flavescens</i>	14474	2.8
	<i>M. fortuitum</i>	6841	3.0
	<i>M. gastri</i>	15754	3.2
	<i>M. gordoneae</i>	14470	3.0
	<i>M. haemophilum</i>	29548	3.0
20	<i>M. intracellulare</i>	13950	3.6
	<i>M. kansasii</i>	12478	3.9
	<i>M. malmoense</i>	29571	2.9
	<i>M. marinum</i>	827	2.9
	<i>M. nonchromogenicum</i>	1930	4.8
	<i>M. phlei</i>	11758	2.9
25	<i>M. scrofulaceum</i>	19981	2.6
	<i>M. shimoidei</i>	27962	3.6
	<i>M. simiae</i>	25275	3.3
	<i>M. smegmatis</i>	e14468	3.0
	<i>M. szulgai</i>	23069	2.8
30	<i>M. terrae</i>	15755	2.8
	<i>M. thermoresistibile</i>	19527	11.7
	<i>M. triviale</i>	23292	3.2
	<i>M. tuberculosis (avirulent)</i>	25177	65.0
	<i>M. tuberculosis (virulent)</i>	27294	53.0
	<i>M. ulcerans</i>	19423	2.5
35	<i>M. vaccae</i>	15483	2.8
	<i>M. xenopi</i>	19971	3.3

Table 19 shows that the probe also did not cross react with RNA from any of the phylogenetically closely related organisms tested by the method just described.

40

45

50

55

TABLE 19

5

HYBRIDIZATION OF THE *M. TUBERCULOSIS* COMPLEX PROBE  
OF EXAMPLE 6 TO PHYLOGENETICALLY CLOSELY RELATED ORGANISMS

	<u>Organism</u>	<u>ATCC #</u>	<u>% Probe Bound</u>
10	<i>Actinomadura madurae</i>	19425	1.3
	<i>Actinoplanes italicus</i>	10049	0.6
	<i>Arthrobacter oxidans</i>	14358	1.1
	<i>Brevibacterium linens</i>	89172	0.8
	<i>Corynebacterium xerosis</i>	373	1.0
15	<i>Dermatophilus congolensis</i>	14367	0.6
	<i>Microbacterium lacticum</i>	8180	1.9
	<i>Nocardia asteroides</i>	19247	0.9
	<i>Nocardia brasiliensis</i>	19296	0.8
	<i>Nocardia otitidis-caviae</i>	14629	1.5
	<i>Nocardiosis dassonvillei</i>	23218	0.5
20	<i>Oerskovia turbata</i>	33225	0.3
	<i>Oerskovia xanthineolytica</i>	27402	0.8
	<i>Rhodococcus aichiensis</i>	33611	1.6
	<i>Rhodococcus aurantiacus</i>	25938	0.7
	<i>Rhodococcus bronchialis</i>	25592	1.5
25	<i>Rhodococcus chubuensis</i>	33609	0.8
	<i>Rhodococcus equi</i>	6939	0.3
	<i>Rhodococcus obuensis</i>	33610	0.8
	<i>Rhodococcus sputi</i>	29627	1.4

30

Example 7

The following additional *Mycobacterium tuberculosis* complex probe also has been identified using a 23S primer with the same sequence as that of Example 6, namely, 5'-CCT GAT TGC CGT CCA GGT TGA GGG AAC CTT TGG G-3':

AGGCACCTGTCCTAAACCCGATTCAAGGGTTC.

This sequence, from 23S rRNA is 31 bases in length and has a *Tm* of 71°C. It hybridizes in the region corresponding to bases 2195-2235 of *E. coli* 23S rRNA. As is the case with the *Mycobacterium tuberculosis* complex probes of Examples 5 and 8 herein, this sequence also was characterized and shown to be specific for the *Mycobacterium tuberculosis* complex of organisms, including *Mycobacterium tuberculosis*, *Mycobacterium africanum* and *Mycobacterium bovis*.

To demonstrate the reactivity and specificity of this probe for *Mycobacterium tuberculosis* complex, it was tested as a probe in hybridization reactions under the conditions described for the probe of Example 5. Table 20 shows that the probe is specific for the *Mycobacterium tuberculosis* complex of organisms.

45

50

55

5 TABLE 20

HYBRIDIZATION OF THE MYCOBACTERIUM TUBERCULOSIS  
COMPLEX PROBE OF EXAMPLE 7 TO MYCOBACTERIAL SPECIES

	<u>Organism</u>	<u>ATCC #</u>	<u>% Probe Bound</u>
10	Mycobacterium africanum	25420	43.0
	M. asiaticum	25274	0.6
	M. avium	25291	0.7
	M. bovis	19210	43.0
15	M. bovis (BCG)	35734	46.0
	M. chelonae	14472	0.6
	M. flavescens	14474	0.6
	M. fortuitum	6841	0.5
	M. gastri	15754	0.9
20	M. gordonae	14470	0.7
	M. haemophilum	29548	0.6
	M. intracellulare	13950	0.6
	M. kansasii	12478	0.9
	M. malmoense	29571	0.8
	M. marinum	827	0.7
25	M. nonchromogenicum	1930	0.8
	M. phlei	11758	0.6
	M. scrofulaceum	19981	0.7
	M. shimoidei	27962	0.8
	M. simiae	25275	0.7
30	M. smegmatis	614468	0.6
	M. szulgai	23069	0.6
	M. tarrae	15755	0.7
	M. thermoresistibile	19527	0.9
	M. triviale	23292	0.7
35	M. tuberculosis (avirulent)	25177	40.0
	M. tuberculosis (virulent)	27294	50.0
	M. ulcerans	19423	0.7
	M. vaccae	15483	0.4
	M. xenopi	19971	0.6

40

Table 21 shows that the probe also did not cross react with RNA from any of the closely related organisms tested by the method just described.

45

50

55

TABLE 21

5 HYBRIDIZATION OF THE M. TUBERCULOSIS COMPLEX PROBE OF  
 EXAMPLE 7 TO PHYLOGENETICALLY CLOSELY RELATED ORGANISMS

	<u>Organism</u>	<u>ATCC #</u>	<u>Probe Bound</u>
10	Actinomadura madurae	19425	1.0
	Actinoplanes italicus	10049	0.6
	Arthrobacter oxidans	14358	0.4
	Brevibacterium linens	e9172	0.8
	Corynebacterium xerosis	373	0.6
	Dermatophilus congolensis	14367	0.8
15	Microbacterium lacticum	8180	0.5
	Nocardia asteroides	19247	0.7
	Nocardia brasiliensis	19296	0.5
	Nocardia otitidis-caviarum	14629	0.6
	Nocardiopsis dassonvillei	23218	0.6
20	Oerskovia turbata	33225	0.8
	Oerskovia xanthineolytica	27402	0.6
	Rhodococcus aichiensis	33611	0.7
	Rhodococcus aurantiacus	25938	0.7
	Rhodococcus bronchialis	25592	0.6
	Rhodococcus chubuensis	33609	0.6
25	Rhodococcus equi	6939	0.6
	Rhodococcus abuensis	33610	0.6
	Rhodococcus sputi	29627	0.9

Notably, overlapping probes may have identical specificity. Compare, for example, the probes of  
 30 Example 6 and 7:

Ex. 6 CTGTCCTAACCCGATTCAAGGGTTCGAGGTTAGATGC  
 Ex. 7 AGGCACTGTCCCTAACCCGATTCAAGGGTTC

There may be several sequences from a particular region which will yield probes with the desired  
 35 hybridization characteristics. In other cases, one probe sequence may be significantly better than another  
 probe differing by a single base. In general, the greater the sequence difference (% mismatch) between a  
 target and nontarget organism, the more likely one will be able to alter the probe without affecting its  
 usefulness for a specific application. This phenomenon also was demonstrated by the derivative probes in  
 Example 3.

In Example 7, five bases were added to the 5' end of the probe in Example 6, and 12 bases were  
 40 removed from the 3' end. The two probes have essentially identical hybridization characteristics.

#### Example 8

45 The Mycobacterium genus is particularly difficult to distinguish from Nocardia, Corynebacterium and  
Rhodococcus. These genera have common antigens, precipitins and G & C counts. Despite the fact that  
 these organisms also exhibit 82-84% rRNA homology to the above listed organisms, we have designed  
 probes which detect all members of the Mycobacterium genus Mycobacterium without cross reacting to  
 the related genera.

50 In addition to the Mycobacterium species probes already disclosed, four probes specific for members  
 of the Mycobacterium genus were identified using one primer complementary to 16S rRNA and one primer  
 complementary to 23S rRNA. Sequence 1 was obtained using a 16S primer with the sequence 5'-TTA CTA  
 GCG ATT CCG ACT TCA-3'. Sequences 2, 3 and 4 were obtained using a 23S primer with the sequence 5'-  
 GTG TCG GTT TTG GGT ACG-3'. Sequence 1 is capable of hybridizing to RNA of the genus Mycobac-  
 terium in the region corresponding to bases 1025-1060 of E. coli 16S rRNA. Sequences 2-4 hybridize in  
 regions corresponding to the following bases of E. coli 23S rRNA in our numbering system (See Figure 2);  
 1440-1475; 1515-1555; 1570-1610 in our numbering system.

The following sequences were characterized and shown to be specific for the genus Mycobacterium:

1. CCA TGC ACC ACC TGC ACA CAG GCC ACA AGG
2. GGC TTG CCC CAG TAT TAC CAC TGA CTG GTA CGG
3. CAC CGA ATT CGC CTC AAC CGG CTA TGC GTC ACC TC
4. GGG GTA CGG CCC GTG TGT GTG CTC GCT AGA GGC

5 Sequence 1, from 16S rRNA, is 30 bases in length and has a Tm of 73°C. Sequence 2, from 23S rRNA, is 33 bases in length and has a Tm of 75°C. Sequence 3, from 23S rRNA, is 35 bases in length and has a Tm of 78°C. Sequence 4, from 23S rRNA, is 33 bases in length and has a Tm of 73°C.

10 To demonstrate the reactivity and specificity of probe 1 for members of the genus Mycobacterium, it was tested as a probe in hybridization reactions under the following conditions. <sup>32</sup>P-labeled oligonucleotide probes were mixed with rRNA released from cells of 30 species of mycobacteria by the sonic disruption techniques described in Murphy et al., U.S. Patent Application Serial No. 841,880. 3×10<sup>7</sup> cells were suspended in 0.1 ml 5% SDS and sonicated for 15 minutes at 50-60°C. One ml of hybridization buffer (45% dodecyl sulfate, 40 mM sodium phosphate pH6.8, 1 mM EDTA, 1 mM EGTA) was added and the mixture incubated at 72°C for 2 hours. Following incubation, 2 g/l of separation solution (containing 15 2.5 g/l cationic magnetic microspheres, 0.17M sodium phosphate buffer pH6.8, 7.5% Triton X-100 (TM), 0.02% sodium azide) was added and incubated at 72°C for 5 minutes. The RNA:probe hybrids, bound to the magnetic particles, were collected and the supernatant removed. One ml wash solution (0.12M sodium phosphate buffer pH6.8, 14% dodecyl sulfate, 5% Triton X-100, 0.02% sodium azide) was added, the particles collected and the supernatant removed. This step was repeated two times. The radioactivity bound to the magnetic particles was determined in a gamma counter. The results are shown in Table 22 and indicate that the probes hybridize to organisms in the genus Mycobacterium and that a combination of probes will detect all members of the genus. Table 23 shows that the probes do not react with other closely related bacteria.

25

TABLE 22

HYBRIDIZATION OF THE MYCOBACTERIUM  
PROBES 1-4 TO MYCOBACTERIAL SPECIES

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe 1 Bound</u>	<u>% Probe 2 Bound</u>	<u>% Probe 3 Bound</u>	<u>% Probe 4 Bound</u>
	<i>Mycobacterium africanum</i>	25420	41.5	14.7	17.9	26.7
35	<i>M. asiaticum</i>	25274	31.8	20.2	7.9	0.1
	<i>M. avium</i>	25291	11.7	34.7	10.1	1.6
	<i>M. bovis</i>	19210	19.4	28.4	44.6	20.9
	<i>M. bovis (BCG)</i>	35734	30.0	35.5	17.8	5.6
	<i>M. chelonae</i>	14472	8.6	0.7	6.3	0.2
40	<i>M. flavescens</i>	14474	29.8	17.7	2.3	0.9
	<i>M. fortuitum</i>	6841	34.7	2.2	4.8	0.2
	<i>M. gastri</i>	15754	27.6	65.1	9.6	22.3
	<i>M. gordoneae</i>	14470	50.7	55.2	3.1	0.4
	<i>M. haemophilum</i>	29548	40.7	60.7	0.4	12.4
45	<i>M. intracellulare</i>	13950	38.8	48.3	0.9	5.4
	<i>M. kansasii</i>	12478	53.4	27.3	24.5	27.8
	<i>M. malmoense</i>	29571	3.1	38.4	0.8	1.5
	<i>M. marinum</i>	827	41.7	4.1	4.8	0.1
	<i>M. non-</i> <i>chromogenicum</i>	1930	35.0	42.9	0.5	16.4
50	<i>M. phlei</i>	11758	23.7	0.6	1.8	0.6
	<i>M. scrofulaceum</i>	19981	35.1	66.9	0.9	26.4
	<i>M. shimoidei</i>	27962	34.6	1.4	1.3	4.8
	<i>M. simiae</i>	25275	45.9	44.0	5.3	0.1
	<i>M. smegmatis</i>	e14468	31.3	4.0	5.6	0.1
	<i>M. szulgai</i>	23069	19.4	22.3	1.5	3.0
55	<i>M. terrae</i>	15755	25.6	21.7	0.4	12.3
	<i>M. thermo-</i> <i>resistibile</i>	19527	20.3	34.5	3.1	17.6

5	<i>M. triviale</i>	23292	37.3	4.6	4.3	0.1
	<i>M. tuberculosis</i> (avirulent)	25177	38.5	26.3	11.3	23.0
	<i>M. tuberculosis</i> (virulent)	27294	13.8	12.4	38.4	22.3
10	<i>M. ulcerans</i>	19423	33.9	28.7	0.4	8.9
	<i>M. vaccae</i>	15483	8.8	36.2	4.8	3.2
	<i>M. xenopi</i>	19971	38.4	2.1	3.8	0.2

15.

TABLE 23

HYBRIDIZATION OF THE MYCOBACTERIUM PROBES  
1-4 TO PHYLOGENETICALLY CLOSELY RELATED ORGANISMS

	Organism	ATCC#	% Probe 1 Bound	% Probe 2 Bound	% Probe 3 Bound	% Probe 4 Bound
	<i>Actinomadura madurae</i>	19425	0.2	0.3	0.2	0.1
25	<i>Actinoplanes italicus</i>	10049	0.4	0.5	0.3	0.2
	<i>Arthrobacter oxidans</i>	14358	0.2	0.4	0.3	0.1
30	<i>Brevibacterium linens</i>	69172	0.3	0.3	0.3	0.1
	<i>Corynebacterium xerosis</i>	373	0.4	0.3	0.3	0.1
	<i>Dermatophilus congolensis</i>	14367	0.4	0.6	0.3	0.2
35	<i>Microbacterium lacticum</i>	8180	0.2	0.3	0.2	0.1
	<i>Nocardia asteroides</i>	19247	0.3	0.3	0.4	0.1
	<i>Nocardia brasiliensis</i>	19296	0.4	0.3	0.6	0.1
40	<i>Nocardia otitidis-caviarum</i>	14629	0.4	0.4	1.0	0.3
	<i>Nocardiopsis dassonvillei</i>	23218	0.3	0.2	0.3	0.1
45	<i>Oerskovia turbata</i>	33225	0.2	0.2	0.3	0.1
	<i>Oerskovia xanthineolytica</i>	27402	0.2	0.3	0.3	0.1
	<i>Rhodococcus aichianensis</i>	33611	0.4	0.2	0.3	0.2
50	<i>Rhodococcus aurantiacus</i>	25938	0.3	0.4	0.3	0.2
	<i>Rhodococcus bronchialis</i>	25592	0.4	0.3	0.3	0.1
	<i>Rhodococcus chubuensis</i>	33609	0.6	0.4	0.3	0.3

55

5	<i>M. triviale</i>	23292	37.3	4.6	4.3	0.1
	<i>M. tuberculosis</i> (avirulent)	25177	38.5	26.3	11.3	23.0
	<i>M. tuberculosis</i> (virulent)	27294	13.8	12.4	38.4	22.3
10	<i>M. ulcerans</i>	19423	33.9	28.7	0.4	8.9
	<i>M. vaccae</i>	15483	8.8	36.2	4.8	3.2
	<i>M. xenopi</i>	19971	38.4	2.1	3.8	0.2

15.

TABLE 23

HYBRIDIZATION OF THE MYCOBACTERIUM PROBES  
1-4 TO PHYLOGENETICALLY CLOSELY RELATED ORGANISMS

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe 1 Bound</u>	<u>% Probe 2 Bound</u>	<u>% Probe 3 Bound</u>	<u>% Probe 4 Bound</u>
	<i>Actinomadura madureae</i>	19425	0.2	0.3	0.2	0.1
25	<i>Actinoplanes italicus</i>	10049	0.4	0.5	0.3	0.2
	<i>Arthrobacter oxidans</i>	14358	0.2	0.4	0.3	0.1
30	<i>Brevibacterium linens</i>	69172	0.3	0.3	0.3	0.1
	<i>Corynebacterium xerosis</i>	373	0.4	0.3	0.3	0.1
	<i>Dermatophilus congolensis</i>	14367	0.4	0.6	0.3	0.2
35	<i>Microbacterium lacticum</i>	8180	0.2	0.3	0.2	0.1
	<i>Nocardia asteroides</i>	19247	0.3	0.3	0.4	0.1
	<i>Nocardia brasiliensis</i>	19296	0.4	0.3	0.6	0.1
40	<i>Nocardia otitidis-caviarum</i>	14629	0.4	0.4	1.0	0.3
	<i>Nocardiopsis dassonvillei</i>	23218	0.3	0.2	0.3	0.1
45	<i>Oerskovia turbata</i>	33225	0.2	0.2	0.3	0.1
	<i>Oerskovia xanthineolytica</i>	27402	0.2	0.3	0.3	0.1
	<i>Rhodococcus aichiensis</i>	33611	0.4	0.2	0.3	0.2
50	<i>Rhodococcus aurantiacus</i>	25938	0.3	0.4	0.3	0.2
	<i>Rhodococcus bronchialis</i>	25592	0.4	0.3	0.3	0.1
	<i>Rhodococcus chubuensis</i>	33609	0.6	0.4	0.3	0.3

55

	Rhodococcus equi	6939	0.4	0.4	0.4	0.5
5	Rhodococcus obuensis	33610	0.5	0.5	0.3	0.1
	Rhodococcus sputi	29627	0.4	0.5	0.4	0.3

10 Example 9

Mycoplasmas are small, aerobic bacteria lacking cell walls. Mycoplasma pneumoniae is estimated to cause 8-15 million infections per year. The infections may be asymptomatic or range in severity from mild to severe bronchitis and pneumonia. The organism is believed to cause about 10% of pneumonias in the general population and 10-60% of the pneumonias of members of groups in prolonged, close contact such as college students and military personnel.

15 Diagnosis until now has required isolation of the organism in culture or demonstration of an increase in antibody titer. Culturing of the organism involves inoculation of respiratory tract specimens onto agar or biphasic media containing bacterial growth inhibitors. Examination for growth at 3-4 and 7-10 days is used 20 to establish the presence or absence of any mycoplasma. Mycoplasma pneumoniae must then be identified by hemadsorption (the ability of M. pneumoniae to adhere sheep or guinea pig erythrocytes), hemolysis (the ability of M. pneumoniae to produce beta hemolysis of sheep or guinea pig erythrocytes in blood agar), growth inhibition by specific antibodies, or immunofluorescence with specific antibodies. The present invention has significant advantages over each of these prior art methods both because of the simplicity of 25 the test and because of the greatly reduced time necessary to achieve a diagnosis.

30 A probe specific for the 5S rRNA of M. pneumoniae was obtained by a comparison of known rRNA sequences. The particular sequences aligned were from M. pneumoniae, M. gallisepticum and Ureaplasma urealyticum (Rogers, M.J. et al. 1985, Proc. Natl. Acad. Sci. USA, 82 (1160-1164), M. capricolum (Hori, H. et al. 1981, Nucl. Acids Res. 9, 5407-5410) and Spiroplasma sp. (Walker, R.T. et al. 1982 Nucl. Acids Res. 10, 6363-6367). The alignments were performed as described above and outlined at page 6. 5S rRNA can be isolated and sequenced as outlined in Rogers et al., or a primer can be made which is complementary to a conserved region in the 5S rRNA and sequencing performed as outlined in Example 1-4. The conserved region of 5S rRNA is documented in Fox, G.E. and Woese, C.R., 1975, Nature 256: 505-507. The following sequence was determined to be specific for Mycoplasma pneumoniae:

35 GCTTGGTGCTTCCTATTCTACTGAAACAGCTACATTGGC.

The sequence is complementary to a unique segment found in the 5S rRNA of Mycoplasma pneumoniae in the region corresponding to bases 65-108 of E. coli 5S rRNA, and was selected by comparison to 5S rRNA sequence from Mycoplasma gallisepticum, Spiroplasma mirum and Ureaplasma urealyticum. The oligonucleotide probe was characterized as described above. The size of the probe was 40 42 bases. The probe has a Tm of 71.5°C.

To demonstrate the reactivity of this sequence for Mycoplasma pneumoniae, the probe was tested in hybridization reactions under the following conditions. <sup>32</sup>P-end-labelled oligonucleotide probe was mixed with 1 microgram ( $7 \times 10^{-13}$  moles) of purified rRNA from Mycoplasma pneumoniae and reacted in 0.12 M PB (equimolar amounts of Na<sub>2</sub>HPO<sub>4</sub> and NaH<sub>2</sub>PO<sub>4</sub>), 1 mM EDTA and 0.2% SDS (sodium dodecyl sulfate) at 45 65°C for 60 minutes in a final volume of 50 microliters. In separate tubes the probe was mixed with the hybridization buffer with and without target Mycoplasma pneumoniae rRNA present. Following separation on hydroxyapatite as outlined previously the hybrids were quantitated by scintillation counting. These results are shown in Table 24.

50

55

TABLE 24

## HYBRIDIZATION OF THE M. PNEUMONIAE 5S rRNA DNA PROBE TO HOMOLOGOUS TARGET rRNAs

	<u>plus rRNA</u>	<u>minus rRNA</u>
10 M. pneumoniae 5S probe	85-95%	0.5%
* % Hybridization = <u>cpm bound to hydroxyapatite</u> total cpm added to reaction		

15 This data shows that the probe has a high extent of reaction to its homologous target and very little non-specific binding to the hydroxyapatite.

Specificity of the M. pneumoniae 5S probe was tested by mixing the <sup>32</sup>P labelled probe with rRNA released from cells from other Mycoplasma species. All hybridization assays were carried out as described in Example 1. Table 25 indicates that the probe is specific for Mycoplasma pneumoniae and does not react with any other Mycoplasma species.

**TABLE 25**

## **HYBRIDIZATION OF M. PNEUMONIAE PROBE TO OTHER MYCOPLASMA SPECIES**

30	<i>Acholeplasma laidlawii</i>	14089	3.3
	<i>M. buccale</i>	23636	1.7
	<i>M. capricolum</i>	23205	2.4
	<i>M. columbinsale</i>	33549	1.4
	<i>M. faucium</i>	25293	1.4
	<i>M. fermentans</i>	15474	1.0
35	<i>M. gallisepticum</i>	19610	1.8
	<i>M. gallopavonis</i>	33551	1.6
	<i>M. genitalium</i>	3353c	1.7
	<i>M. hominis</i>	14027	1.3
	<i>M. orale</i>	23714	1.8
40	<i>M. pneumoniae</i>	15531	78.0
	<i>M. primatum</i>	15497	1.6
	<i>M. salivarium</i>	23064	0.6
	<i>Spiroplasma mirum</i>		2.3

As shown in Table 26, the probe did not react with any other closely related or phylogenetically diverse species of bacteria.

5

**TABLE 27**  
**HYBRIDIZATION OF MYCOPLASMA PNEUMONIAE**  
**PROBES 2-5 TO MYCOPLASMA SPECIES**

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
10	Acholeplasma axanthum	27378	0.34
	Acholeplasma laidlawii	14089	0.30
	Mycoplasma arginini	23838	0.20
	Mycoplasma arthritidis	19611	0.49
15	Mycoplasma bovigenitalium	19852	0.18
	Mycoplasma bovis	25523	0.43
	Mycoplasma buccale	23636	0.37
	Mycoplasma californicum	33451	0.79
	Mycoplasma capricolum	23205	0.38
20	Mycoplasma columbinasale	33549	0.54
	Mycoplasma columborale	29258	0.50
	Mycoplasma faecium	25293	0.45
	Mycoplasma fermentans	15474	0.27
	Mycoplasma gallisepticum	19610	0.25
	Mycoplasma gallopavonis	33551	0.47
25	Mycoplasma genitalium	33530	2.5
	Mycoplasma hominis	14027	0.52
	Mycoplasma hyorhinis	17981	0.46
	Mycoplasma orale	23714	0.56
	Mycoplasma pneumoniae	15531	34.0
	Mycoplasma primatum	15497	0.71
30	Mycoplasma pulmonis	19612	0.68
	Mycoplasma salivarium	23064	0.46
	Spiroplasma citri	29416	0.60
	Spiroplasma mirum	29335	0.52

35

**TABLE 28**  
**HYBRIDIZATION OF MYCOPLASMA PNEUMONIAE**  
**PROBES 2-5 WITH OTHER BACTERIA**

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
40	Actinomyces israelii	10049	1.0
	Bacteroides fragilis	23745	1.4
	Bifidobacterium breve	15700	1.0
	Bordetella bronchiseptica	10580	0.9
45	Clostridium innocuum	14501	1.0
	Clostridium pasteurianum	6013	0.9
	Clostridium perfringens	13124	1.1
	Clostridium ramosum	25582	1.0
	Corynebacterium xerosis	373	0.8
50	Erysipelothrix rhusiopathiae	19414	1.1
	Escherichia coli	11775	1.0
	Haemophilus influenzae	19418	0.9
	Klebsiella pneumoniae	15531	1.0
	Lactobacillus acidophilus	4356	1.4
	Legionella pneumophila	33154	0.8
55	Listeria monocytogenes	15313	1.2

5

**TABLE 27**  
**HYBRIDIZATION OF MYCOPLASMA PNEUMONIAE**  
**PROBES 2-5 TO MYCOPLASMA SPECIES**

	<b>Organism</b>	<b>ATCC#</b>	<b>% Probe Bound</b>
10	Acholeplasma axanthum	27378	0.34
	Acholeplasma laidlawii	14089	0.30
	Mycoplasma arginini	23838	0.20
	Mycoplasma arthritidis	19611	0.49
15	Mycoplasma bovigenitalium	19852	0.18
	Mycoplasma bovis	25523	0.43
	Mycoplasma buccale	23636	0.37
	Mycoplasma californicum	33451	0.79
	Mycoplasma capricolum	23205	0.38
	Mycoplasma columbinasale	33549	0.54
20	Mycoplasma columborale	29258	0.50
	Mycoplasma faecium	25293	0.45
	Mycoplasma fermentans	15474	0.27
	Mycoplasma gallisepticum	19610	0.25
	Mycoplasma gallopavonis	33551	0.47
25	Mycoplasma genitalium	33530	2.5
	Mycoplasma hominis	14027	0.52
	Mycoplasma hyorhinis	17981	0.46
	Mycoplasma orale	23714	0.56
	Mycoplasma pneumoniae	15531	34.0
	Mycoplasma primatum	15497	0.71
30	Mycoplasma pulmonis	19612	0.68
	Mycoplasma salivarium	23064	0.46
	Spiroplasma citri	29416	0.60
	Spiroplasma mirum	29335	0.52

35

**TABLE 28**  
**HYBRIDIZATION OF MYCOPLASMA PNEUMONIAE**  
**PROBES 2-5 WITH OTHER BACTERIA**

	<b>Organism</b>	<b>ATCC#</b>	<b>% Probe Bound</b>
40	Actinomyces israelii	10049	1.0
	Bacteroides fragilis	23745	1.4
	Bifidobacterium breve	15700	1.0
	Bordetella bronchiseptica	10580	0.9
45	Clostridium innocuum	14501	1.0
	Clostridium pasteurianum	6013	0.9
	Clostridium perfringens	13124	1.1
	Clostridium ramosum	25582	1.0
	Corynebacterium xerosis	373	0.8
50	Erysipelothrix rhusiopathiae	19414	1.1
	Escherichia coli	11775	1.0
	Haemophilus influenzae	19418	0.9
	Klebsiella pneumoniae	15531	1.0
	Lactobacillus acidophilus	4356	1.4
	Legionella pneumophila	33154	0.8
55	Listeria monocytogenes	15313	1.2

	<i>Moraxella osloensis</i>	19976	1.1
5	<i>Mycobacterium tuberculosis</i>	25177	1.0
	<i>Neisseria meningitidis</i>	13077	1.0
	<i>Pasteurella multocida</i>	6529	1.6
	<i>Peptococcus magnus</i>	14955	0.9
	<i>Propionibacterium acnes</i>	6919	1.1
	<i>Pseudomonas aeruginosa</i>	25330	1.0
10	<i>Staphylococcus aureus</i>	12600	1.0
	<i>Streptococcus faecalis</i>	19433	1.5
	<i>Streptococcus mitis</i>	9811	1.0
	<i>Streptococcus pneumoniae</i>	6306	1.0
	<i>Streptococcus pyogenes</i>	19615	1.1

15

Example 10

The genus *Legionella* contains 22 species which are all potentially pathogenic for humans. These organisms cause Legionnaires' disease, an acute pneumonia, or Pontiac fever, an acute, non-pneumonic, febrile illness that is not fatal.

*Legionella* species have also been shown to be responsible for nosocomial pneumonia occurring predominantly among immunocompromised patients.

Legionellosis, which includes Legionnaires' disease and Pontiac fever, is diagnosed on the basis of clinical symptoms, either direct or indirect fluorescence antibody tests, and by culture using a buffered charcoal yeast extract (BCYE) agar containing selective antimicrobial agents. There is no single definitive genus test known in the prior art. (See Bergey's Manual of Systematic Bacteriology at page 283, (ed. 1984)). The fluorescent antibody tests are not able to identify all species of *Legionella*, but only those few for which antibodies exist. The culture method is not definitively diagnostic for *Legionella* species.

30 The oligonucleotide sequences described below, when used as probes in a nucleic acid hybridization assay, accurately identify all species of *Legionella*. This assay is more sensitive than culture or antibody tests and shortens significantly the time of identification and, thus, diagnosis. The assay, therefore, represents a significant improvement over prior diagnostic methods.

Three probe sequences specific for the genus *Legionella* were obtained by utilizing three unique primers complementary to conserved regions on both 16S and 23S rRNA. Sequence 1 was obtained by using a 16S primer with the sequence 5'-TCT ACG CAT TTC ACC GCT ACA C-3'. Probe sequence 2 was obtained with a 23S primer of sequence 5'-CAG TCA GGA GTA TTT AGC CTT-3'. Probe sequence 3 was obtained with a 16S primer of sequence 5'GCT CGT TGC GGG ACT TAA CCC ACC AT-3'. Sequencing with these primers was performed as described for previous examples.

40 The following three sequences were characterized by the criteria described in Example 1 and were shown to be specific for the genus *Legionella*. The phylogenetically nearest neighbors *Escherichia coli*, *Pseudomonas aeruginosa*, *Vibrio parahaemolyticus* and *Acinetobacter calcoaceticus* were used as comparisons with sequences from *Legionella* species.

- 1. TACCCCTCTCCCATACTCGAGTCACCGAGTATTATCTGACC
- 45 2. GGATTTCACGTGTCCCGCCTACTTGTTCGGGTGCGTAGTTC
- 3. CATCTCTGCAAATTCACTGTATGTCAAGGGTAGGTAAGG. Sequence 1, from 16S rRNA, is 40 bases in length and has a Tm of 72°C. Sequence 2, from 23S rRNA, is 42 bases in length and has a Tm of 73°C. Sequence 3, from 16S rRNA, is 40 bases in length and has a Tm of 68°C. These sequences are capable of hybridizing to RNA of the genus *Legionella* in the regions corresponding respectively to, 630-675 bases of *E. coli* 16s rRNA; 350-395 of *E. coli* 23s rRNA; and 975-1020 of *E. coli* 16s rRNA. When mixed together the probes had a combined average Tm of 73°C. Analysis on polyacrylamide gels showed that each probe was the correct length and sequence analysis demonstrated that each was the correct sequence of bases.

When the three probes were mixed and used in a hybridization assay, they were found to be specific for the genus *Legionella* (Tables 29 and 30) and did not cross react with other respiratory pathogens or with any selected organism from the phylogenetic tree (Tables 31 and 32). Use of more than one probe, i.e., a mixture of probes, can result in increased assay sensitivity and/or in an increase in the number of non-viral organisms to be detected.

5

10

15

TABLE 29

HYBRIDIZATION OF LEGIONELLA  
PROBES TO HOMOLOGOUS TARGET rRNA

<u>Legionella probe</u>	<u>plus rRNA</u>	<u>minus rRNA</u>
	80%	1.0%

TABLE 30

HYBRIDIZATION OF LEGIONELLA  
PROBES TO LEGIONELLA SPECIES

<u>Organism</u>	<u>ATCC#</u>	<u># Probes Bound</u>
L. anisa	35292	42.0
L. bozemani	33217	58.0
L. cherrii	35252	69.0
L. dumoffii	33279	57.0
L. erythra	CDC#9P1W044C	26.0
L. feeleii	35303	59.0
L. hackeliae	35250	47.0
L. jamestowniensis	35298	20.0
L. jordanis	33623	50.6
L. longbeachae	33484	48.0
L. maceachernii	35300	25.0
L. mieddadei	33704	38.0
L. oakridgensis	33761	44.0

45

50

55

5	<i>L. parisiensis</i>	9060	69.0
	<i>L. pneumophila</i> 1*	6736	75.0
	" 2		64.0
	" 3		73.0
	" 4		73.0
	" 5		78.0
10	" 6		75.0
	" 7		73.0
	" 8		63.0
	" 11		75.0
	<i>L. rubrilucens</i>	35304	12.0
	<i>L. sainthelensi</i>	35248	61.0
15	<i>L. sainticrucis</i>	35301	24.0
	<i>L. spiritansis</i>	CDC#MSH9	55.0
	<i>L. steigerwaltii</i>	7430	56.0
	<i>L. wadsworthii</i>	33877	37.0
20	* The numbers 1-8 and 11 are serotypes of <i>L. pneumophila</i> .		

TABLE 31  
HYBRIDIZATION OF LEGIONELLA PROBES TO  
RESPIRATORY PATHOGENS

	<u>Organisms</u>	<u>ATCC#</u>	<u># Probe Bound</u>
30	<i>Corynebacterium xerosis</i>	373	2.1
	<i>Haemophilus influenzae</i>	19418	2.3
	<i>Klebsiella pneumoniae</i>	23357	2.0
	<i>Mycoplasma pneumoniae</i>	19531	2.3
	<i>Neisseria meningitidis</i>	13090	2.2
35	<i>Pseudomonas aeruginosa</i>	25330	1.2
	<i>Propionibacterium acnes</i>	6919	1.6
	<i>Streptococcus pneumoniae</i>	6306	0.8
	<i>Staphylococcus aureus</i>	25923	1.6

TABLE 32  
HYBRIDIZATION OF LEGIONELLA PROBES TO  
A PHYLOGENETIC CROSS SECTION OF BACTERIAL SPECIES

	<u>Organisms</u>	<u>ATCC#</u>	<u># Probe Bound</u>
	<i>Acinetobacter calcoaceticus</i>	33604	1.4
	<i>Branhamella catarrhalis</i>	25238	2.0
	<i>Bacillus subtilis</i>	6051	1.9
50	<i>Bacteroides fragilis</i>	23745	2.2
	<i>Campylobacter jejuni</i>	33560	1.2
	<i>Chromobacterium violaceum</i>	29094	1.3
	<i>Clostridium perfringens</i>	13124	1.9
	<i>Deinococcus radiodurans</i>	35073	1.8
	<i>Darxia gummosa</i>	15994	2.0
55	<i>Enterobacter aerogenes</i>	13948	1.4

	<i>Escherichia coli</i>	11'75	1.2
	<i>Mycoplasma hominis</i>	14027	1.1
	<i>Proteus mirabilis</i>	29H06	1.4
5	<i>Pseudomonas cepacia</i>	11762	1.1
	<i>Rahnella aquatilis</i>	33071	1.7
	<i>Rhodospirillum rubrum</i>	11170	2.0
	<i>Streptococcus mitis</i>	9811	2.0
	<i>Vibrio parahaemolyticus</i>	17802	2.0
	<i>Yersinia enterocolitica</i>	9610	1.2

10

Three additional probe sequences (numbered 4-6) specific for the genus Legionella were obtained by utilizing two primers complementary to conserved regions on 23S rRNA. Sequence 4 was made from a 23S primer with the sequence 5'-CCT TCT CCC GAA GTT ACG G-3'. Probe sequences 5 and 6 were made from a 23S primer of sequence 5'-AAG CCG GTT ATC CCC GGG GTA ACT TTT-3'. Sequencing with these primers was performed as described for previous examples.

The following three sequences were characterized by the criteria previously described and were shown to be specific for the genus Legionella. The phylogenetically nearest neighbors Escherichia coli, Pseudomonas aeruginosa, Vibrio parahaemolyticus and Actinobacter calcoaceticus were used for comparisons with sequences from Legionella species.

20 4. GCG GTA CGG TTC TCT ATA AGT TAT GGC TAG C

5. GTA CCG AGG GTA CCT TTG TGC T

6. CAC TCT TGG TAC GAT GTC CGA C

Probe 4, complementary to 23S rRNA in the region corresponding to bases 1585-1620 of E. coli 23S rRNA, is 31 bases long and has a Tm of 67°C. Probe 5, complementary to 23S rRNA in the region corresponding to bases 2280-2330 of E. coli 23s rRNA, is 22 bases long and has a Tm of 66°C. Probe 6, complementary to 23S rRNA in the same region as Probe 5, is 22 bases long and has a Tm of 63°C.

When the three probes were mixed with probe 3 above and used in a hybridization assay as described for probes 1-3, they were found to be specific for the genus Legionella (Table 33) and did not cross react with other respiratory pathogens or with any selected organism from the phylogenetic tree (Tables 34 and 35). Using more than one probe, i.e., a mixture of probes, can improve assay sensitivity and/or increase the number of non-viral organisms detected.

35

40

45

50

55

	<i>Escherichia coli</i>	11'75	1.2
	<i>Mycoplasma hominis</i>	14027	1.1
	<i>Proteus mirabilis</i>	29'06	1.4
5	<i>Pseudomonas cepacia</i>	11762	1.1
	<i>Rahnella aquatilis</i>	33071	1.7
	<i>Rhodospirillum rubrum</i>	11170	2.0
	<i>Streptococcus mitis</i>	9811	2.0
	<i>Vibrio parahaemolyticus</i>	17802	2.0
	<i>Yersinia enterocolitica</i>	9610	1.2

10

Three additional probe sequences (numbered 4-6) specific for the genus Legionella were obtained by utilizing two primers complementary to conserved regions on 23S rRNA. Sequence 4 was made from a 23S primer with the sequence 5'-CCT TCT CCC GAA GTT ACG G-3'. Probe sequences 5 and 6 were made from a 23S primer of sequence 5'-AAG CCG GTT ATC CCC GGG GTA ACT TTT-3'. Sequencing with these primers was performed as described for previous examples.

The following three sequences were characterized by the criteria previously described and were shown to be specific for the genus Legionella. The phylogenetically nearest neighbors Escherichia coli, Pseudomonas aeruginosa, Vibrio parahaemolyticus and Actinobacter calcoaceticus were used for comparisons with sequences from Legionella species.

20 4. GCG GTA CGG TTC TCT ATA AGT TAT GGC TAG C

5. GTA CCG AGG GTA CCT TTG TGC T

6. CAC TCT TGG TAC GAT GTC CGA C

Probe 4, complementary to 23S rRNA in the region corresponding to bases 1585-1620 of E. coli 23S rRNA, is 31 bases long and has a Tm of 67°C. Probe 5, complementary to 23S rRNA in the region corresponding to bases 2280-2330 of E. coli 23s rRNA, is 22 bases long and has a Tm of 66°C. Probe 6, complementary to 23S rRNA in the same region as Probe 5, is 22 bases long and has a Tm of 63°C.

When the three probes were mixed with probe 3 above and used in a hybridization assay as described for probes 1-3, they were found to be specific for the genus Legionella (Table 33) and did not cross react with other respiratory pathogens or with any selected organism from the phylogenetic tree (Tables 34 and 35). Using more than one probe, i.e., a mixture of probes, can improve assay sensitivity and/or increase the number of non-viral organisms detected.

35

40

45

50

55

5

10

16

TABLE 33

HYBRIDIZATION OF LEGIONELLA PROBES TO  
LEGIONELLA SPECIES

	<u>Organism</u>	<u>ATCC#</u>	<u># Probes Bound</u>
	L. anisa	35292	29.6
	L. bozemanii	33217	35.5
	L. cherrii	35252	29.2
26	L. dumoffii	33279	26.0
	L. erythra	35303	32.0
	L. feillie	CDC#9P1W044C	32.0
	L. hackeliae	35250	39.0
	L. jamastowniensis	35298	31.2
30	L. jordanis	33623	25.7
	L. longbeachae	33484	27.6
	L. maceahernii	35300	39.3
	L. micdadei	33204	31.0
	L. oakridgensis	33761	24.4
	L. parisiensi	35299	31.2
35	L. pneumophila 1*	33153	40.0
	" 2	33154	38.5
	" 3	33155	44.6
	" 4	33156	48.6
	" 5	33216	32.0
	" 6	33215	43.0
40	" 7	33823	29.5
	" 8	35096	37.6
	" 11	43130	44.5
	L. rubrilucens	35304	30.1
	L. sainthalensis	35248	27.0
45	L. sainticrucis	35301	22.0

50

55

5	L. spiritensis	CDC#M3H9	40.5
	L. steigerwaltii	35302	31.7
	L. wadsworthii	33877	30.0

10 \* The numbers 1-8 and 11 are serotypes of L. pneumophilia.

---

15 TABLE 34

HYBRIDIZATION OF LEGIONELLA PROBES TO  
RESPIRATORY PATHOGENS

	<u>Organisms</u>	<u>ATCC#</u>	<u># Probe Bound</u>
20	Corynebacterium xerosis	373	0.13
	Baemophilum influenzae	19418	0.12
	Klebsiella pneumoniae	23357	0.13
	Neisseria meningitidis	13090	0.14
25	Pseudomonas aeruginosa	25330	0.13
	Propionibacterium acnes	6919	0.11
	Streptococcus pneumoniae	6306	0.08
	Staphylococcus auerus	25923	0.15

---

30 TABLE 35

HYBRIDIZATION OF LEGIONELLA PROBES TO  
A PHYLOGENETIC CROSS SECTION OF BACTERIAL SPECIES

	<u>Organisms</u>	<u>ATCC#</u>	<u># Probe Bound</u>
	Acinetobacter calcoaceticus	33604	0.12
	Branhamella catarrhalis	25238	0.13
	Bacillus subtilis	6051	0.09
40	Bacteroides fragilis	23745	0.12
	Campylobacter jejuni	33560	0.06
	Chromobacterium violaceum	29094	0.33
	Clostridium perfringens	13124	0.07
	Deinococcus radiodurans	35073	0.11
	Derxia gummosa	15994	0.15
45	Enterobacter aerogenes	13048	0.26
	Escherichia coli	11775	0.09
	Mycoplasma hominis	14027	0.09
	Proteus mirabilis	29906	0.09
	Pseudomonas cepacia	17762	0.20
50	Rahnella aquatilis	33071	0.15
	Rhodospirillum rubrum	11170	0.13
	Streptococcus mitis	9811	0.07
	Vibrio parahaemolyticus	17802	0.11
	Yersinia enterocolitica	9610	0.19

---

5	L. <i>spiritensis</i>	CDC#MSH9	40.5
	L. <i>steigerwaltii</i>	35302	31.7
	L. <i>wadsworthii</i>	33877	30.0

10 \* The numbers 1-8 and 11 are serotypes of *L. pneumophilia*.

---

15 TABLE 34

HYBRIDIZATION OF LEGIONELLA PROBES TO  
RESPIRATORY PATHOGENS

	<u>Organisms</u>	<u>ATCC#</u>	<u># Probe Bound</u>
20	<i>Corynebacterium xerosis</i>	373	0.13
	<i>Haemophilus influenzae</i>	19418	0.12
	<i>Klebsiella pneumoniae</i>	23357	0.13
	<i>Neisseria meningitidis</i>	13090	0.14
25	<i>Pseudomonas aeruginosa</i>	25330	0.13
	<i>Propionibacterium acnes</i>	6919	0.11
	<i>Streptococcus pneumoniae</i>	6306	0.08
	<i>Staphylococcus auer</i> us	25923	0.15

---

30 TABLE 35

HYBRIDIZATION OF LEGIONELLA PROBES TO  
A PHYLOGENETIC CROSS SECTION OF BACTERIAL SPECIES

	<u>Organisms</u>	<u>ATCC#</u>	<u># Probe Bound</u>
	<i>Acinetobacter calcoaceticus</i>	33604	0.12
	<i>Branhamella catarrhalis</i>	25238	0.13
	<i>Bacillus subtilis</i>	6051	0.09
40	<i>Bacteroides fragilis</i>	23745	0.12
	<i>Campylobacter jejuni</i>	33560	0.06
	<i>Chromobacterium violaceum</i>	29094	0.33
	<i>Clostridium perfringens</i>	13124	0.07
	<i>Dainococcus radiodurans</i>	35073	0.11
	<i>Derxia gummosa</i>	15994	0.15
45	<i>Enterobacter aerogenes</i>	13048	0.26
	<i>Escherichia coli</i>	11775	0.09
	<i>Mycoplasma hominis</i>	14027	0.09
	<i>Proteus mirabilis</i>	29906	0.09
	<i>Pseudomonas cepacia</i>	17762	0.20
50	<i>Rahnella aquatilis</i>	33071	0.15
	<i>Rhodospirillum rubrum</i>	11170	0.13
	<i>Streptococcus mitis</i>	9811	0.07
	<i>Vibrio parahaemolyticus</i>	17802	0.11
	<i>Yersinia enterocolitica</i>	9610	0.19

---

counting. Table 36 shows that probes 1 and 2 hybridize well to all serotypes of C. trachomatis tested. Probe 1 does not react with any strain of C. psittaci tested and probe 2 does not react with two of the strains. Probe 2 does react with the ovine polyarthritis strain of C. psittaci, an organism which is not known to infect humans. Tables 37 demonstrates the reactivity and specificity of probes 3-9 when <sup>125</sup>I-labeled and used as a mix. In this case, the hybrids were bound to cationic magnetic particles as described in Arnold et al., U.S. Patent App. Ser. No. 020,888 filed March 2, 1987. These probes hybridize well to all strains of C. trachomatis tested and not to any strains of C. psittaci. Probes 3-9 were further tested against a panel of organisms commonly found in the urogenital tract (Table 38) and a phylogenetic cross section of organisms (Table 39). In all cases, the probes were shown to be specific. Probe 10 is 25% non-homologous to C. psittaci and also should be specific for C. trachomatis.

15

20

25

TABLE 36

HYBRIDIZATION OF CHLAMYDIA TRACHOMATIS PROBES 1 AND 2  
TO CHLAMYDIA RNA

Organism	ATCC#	<u># Probe Bound</u>	
		Probe 1	Probe 2
Chlamydia trachomatis serotype C	VR578	22	39
Chlamydia trachomatis serotype E	VR348B	27	48
Chlamydia trachomatis serotype G	VR878	20	44
Chlamydia trachomatis serotype I	VR880	20	42
Chlamydia trachomatis serotype K	VR887	28	45
Chlamydia psittaci guinea pig conjunctivitis strain	VR813	1.2	1.4
Chlamydia psittaci ovine abortion strain	VR656	1.0	3.0
Chlamydia psittaci ovine poly- arthritis strain	VR619	1.1	35.3

45

50

55

TABLE 37

5

HYBRIDIZATION OF CHLAMYDIA TRACHOMATIS PROBES 3-9  
WITH CHLAMYDIA rRNA

10	Organism	Serovar	ATCC#	Ratio Counts	
					Bound*
	C. trachomatis	A		689	
	C. trachomatis	B		560	
	C. trachomatis	Ba		1066	
15	C. trachomatis	C	VR548	962	
	C. trachomatis	D		1192	
	C. trachomatis	E	VR348	1022	
	C. trachomatis	F		391	
	C. trachomatis	G	VR878	874	
	C. trachomatis	H		954	
20	C. trachomatis	I	VR880	943	
	C. trachomatis	J		482	
	C. trachomatis	K	VR887	999	
	C. trachomatis	L1		638	
	C. trachomatis	L2		501	
	C. trachomatis	L3	VR903	821	
25	C. psittaci		VR125	1.6	
	C. psittaci		VR629	0.9	
	C. psittaci		VR656	1.3	
	C. psittaci		VR813	1.2	

30 \*Ratio = counts bound when RNA present  
counts bound when no RNA present

TABLE 38

35

HYBRIDIZATION OF CHLAMYDIA TRACHOMATIS PROBES 3-9  
TO ORGANISMS FOUND IN THE UROGENITAL TRACT.

40	Organism	ATCC#	Ratio Counts	
				Bound*
	Achromobacter xylosoxidans	27061		1.9
	Acinetobacter lwoffii	15309		1.2
	Branhamella catarrhalis	25238		1.2
45	Candida albicans	18804		2.4
	Flavobacterium meningosepticum	13253		1.1
	Gardnerella vaginalis	14018		1.3
	Lactobacillus acidophilus	4356		0.8
	Listeria monocytogenes	15313		0.7
	Mycobacterium smegmatis	14468		1.1
50	Moraxalla osloensis	19976		1.3
	Neisseria gonorrhoeae	19424		2.3
	Pasteurella multocida	6529		1.0
	Peptostreptococcus anaerobius	27337		1.2
	Streptococcus agalactiae	13813		4.0
55	Streptococcus faecalis	19433		2.6

\*Ratio = counts bound when RNA present  
counts bound when no RNA present

5

TABLE 39

10 HYBRIDIZATION OF CHLAMYDIA TRACHOMATIS PROBES 3-9  
 TO PHYLOGENETICALLY DIVERSE ORGANISMS.

Organism	ATCC#	Ratio Counts Bound*
Bacillus subtilis	6051	2.2
Bacteroides fragilis	23745	1.6
Campylobacter jejuni	33560	1.4
Chromabacterium violaceum	29094	1.4
Deinococcus radiodurans	35073	1.8
Dexia gummosa	15994	1.3
Enterobacter aerogenes	13048	1.9
Escherichia coli	11775	1.9
Mycoplasma hominis	14027	1.3
Pseudomonas cepacia	17762	2.2
Proteus mirabilis	29906	2.2
Rahnella aquatilis	33071	1.9
Rhodospirillum rubrum	11170	1.9
Vibrio parahaemolyticus	17802	2.0
Yersinia enterocolitica	9610	2.5

30 \*Ratio = counts bound when RNA present  
counts bound when no RNA present

35 Example 12

Campylobacters are motile, microaerophilic, gram negative curved rods. The genus is quite diverse and distinct from other genera. Although the genus is well defined, some revision is occurring at the species level (Romanuk, P.J. et al. J. Bacteriol. 169:2137-2141 (1987). Three Campylobacter species, Campylobacter jejuni, C. coli and C. lalandi, cause enteritis in humans. The disease includes diarrhea, fever, nausea, abdominal pain and in some cases, vomiting. These organisms cause an estimated 2 million infections per year in the United States (estimate based on the number of Salmonella and Shigella induced cases of diarrheal disease). Other members of the genus cause septicemias in humans and abortion and infertility in sheep and cattle.

45 Diagnosis of Campylobacter enteritis is currently dependent upon growth and isolation of the organism in culture, followed by a number of biochemical tests. Optimum growth of campylobacters requires special conditions such as low oxygen tension and high temperature (42°C). No single set of conditions is recommended for isolation of all Campylobacter species.

The oligonucleotide sequences listed below, when used in a hybridization assay, hybridize to the 16S rRNA of the Campylobacter species of interest. The present invention has significant advantages over the prior art methods of detection of Campylobacter because one probe can detect all Campylobacters of interest; the other two probes detect the enteric Campylobacters and one can detect human isolates of Campylobacter. In addition, the probes have advantages over the prior art in terms of ease of the assay and greatly reduced time to identification and therefore, diagnosis.

55 The four probes which hybridize to the 16S rRNA of Campylobacter species of interest were constructed using three unique primers complementary to 16S rRNA. Sequences 1 and 2 were made using a 16S primer with the sequences 5'-GTA TTA CCG CGG CTG CTG GCA C-3'. Sequence 3 was made using a 16S primer with the sequence 5'-CCG CTT GTG CGG GCC CCC GTC AAT TC-3'. Sequence 4 was made

\*Ratio = counts bound when RNA present  
counts bound when no RNA present

5

TABLE 39

10 HYBRIDIZATION OF CHLAMYDIA TRACHOMATIS PROBES 3-9  
 TO PHYLOGENETICALLY DIVERSE ORGANISMS.

Organism	ATCC#	Ratio Counts Bound*
Bacillus subtilis	6051	2.2
Bacteroides fragilis	23745	1.6
Campylobacter jejuni	33560	1.4
Chromabacterium violaceum	29094	1.4
Deinococcus radiodurans	35073	1.8
Dexia gummosa	15994	1.3
Enterobacter aerogenes	13048	1.9
Escherichia coli	11775	1.9
Mycoplasma hominis	14027	1.3
Pseudomonas cepacia	17762	2.2
Proteus mirabilis	29906	2.2
Rahnella aquatilis	33071	1.9
Rhodospirillum rubrum	11170	1.9
Vibrio parahaemolyticus	17802	2.0
Yersinia enterocolitica	9610	2.5

30 \*Ratio = counts bound when RNA present  
counts bound when no RNA present

35 Example 12

Campylobacters are motile, microaerophilic, gram negative curved rods. The genus is quite diverse and distinct from other genera. Although the genus is well defined, some revision is occurring at the species level (Romanuk, P.J. et al., *J. Bacteriol.* 169:2137-2141 (1987). Three *Campylobacter* species, *Campylobacter jejuni*, *C. coli* and *C. lari*, cause enteritis in humans. The disease includes diarrhea, fever, nausea, abdominal pain and in some cases, vomiting. These organisms cause an estimated 2 million infections per year in the United States (estimate based on the number of *Salmonella* and *Shigella* induced cases of diarrheal disease). Other members of the genus cause septicemias in humans and abortion and infertility in sheep and cattle.

45 Diagnosis of *Campylobacter* enteritis is currently dependent upon growth and isolation of the organism in culture, followed by a number of biochemical tests. Optimum growth of campylobacters requires special conditions such as low oxygen tension and high temperature (42°C). No single set of conditions is recommended for isolation of all *Campylobacter* species.

The oligonucleotide sequences listed below, when used in a hybridization assay, hybridize to the 16S rRNA of the *Campylobacter* species of interest. The present invention has significant advantages over the prior art methods of detection of *Campylobacter* because one probe can detect all *Campylobacters* of interest; the other two probes detect the enteric *Campylobacters* and one can detect human isolates of *Campylobacter*. In addition, the probes have advantages over the prior art in terms of ease of the assay and greatly reduced time to identification and therefore, diagnosis.

55 The four probes which hybridize to the 16S rRNA of *Campylobacter* species of interest were constructed using three unique primers complementary to 16S rRNA. Sequences 1 and 2 were made using a 16S primer with the sequences 5'-GTA TTA CCG CGG CTG CTG GCA C-3'. Sequence 3 was made using a 16S primer with the sequence 5'-CCG CTT GTG CGG GCC CCC GTC AAT TC-3'. Sequence 4 was made

the genus Pseudomonas are particularly important to identify in a clinical sample because of the resistance of the organisms to antibiotics. Nucleic acid homology studies have divided the genus into five homology classes known as RNA groups I-V. Eighty-three percent of all clinical isolates of Pseudomonas are from RNA group I and Pseudomonas aeruginosa is by far the most common species isolated.

5 Current methods of detection of pseudomonas require culture of a patient sample for 24-72 hours, followed by a battery of biochemical tests. The oligonucleotide sequence below, when used in a hybridization assay, detects the clinically important group I pseudomonas. The present invention reduces the number of tests which must be run on a sample, and reduces the time to detection. This represents a significant improvement over prior art methods.

10 The sequence was obtained with a primer complementary to a conserved region on 23S rRNA with the sequence 5'-CTT TCC CTC ACG GTA-3'. The following sequence was shown to detect group I pseudomonads:

1. CAG ACA AAG TTT CTC GTG CTC CGT CCT ACT CGA TT

The probe is 35 bases in length and has a Tm of 70°C. It is capable of hybridizing to the RNA of group 15 I Pseudomonas in the region corresponding to bases 385-405 of E. coli 23s rRNA. To demonstrate the reactivity and specificity of the probe, it was used in a hybridization assay. <sup>32</sup>P-end-labeled oligonucleotide was mixed with RNA released from at least 10<sup>7</sup> organisms by standard methods in 0.48 M sodium phosphate pH 6.8, 1% sodium dodecyl sulfate, 1 mM EDTA, 1 mM EGTA and incubated at 65°C for two hours. After incubation, the RNA:DNA hybrids were bound to hydroxyapatite as described for previous

20 examples and the radioactivity bound was determined by scintillation counting. Table 44 demonstrates that the probe reacted well with all 8 species of group I pseudomonads that were tested. The probe did not react with RNA from group II or group V organisms. A low reaction was seen with Pseudomonas acidovorans, a group III organism which represents < 1% of all isolates of nonfermentative bacilli from clinical samples. Table 45 demonstrates that the probe does not react with other closely related organisms 25 which were tested.

30

35

40

45

50

55

sample, the time to identification and therefore, diagnosis, and thus represents a significant improvement over prior art methods.

The probe specific for Enterobacter cloacae was obtained with a primer complementary to a conserved region of 23S rRNA with the sequence 5'-CAG TCA GGA GTA TTT AGC CTT-3'.

5 The following sequence was characterized and shown to be specific for E. cloacae. The phylogenetically nearest neighbors Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris, Salmonella enteritidis, and Citrobacter freundii were used as comparisons with the sequence of E. cloacae.

1. GTG TGT TTT CGT GTA CGG GAC TTT CAC CC

10 The probe is 29 bases in length and has a Tm of 68°C. It is capable of hybridizing to RNA of E. cloacae in the region corresponding to bases 305-340 of E. coli 23s rRNA. To demonstrate the reactivity and specificity of the probe for E. cloacae, it was used in a hybridization assay. <sup>32</sup>P-end-labeled oligonucleotide probe was mixed with RNA released from at least 10<sup>7</sup> organisms in 1% sodium dodecyl sulfate, 0.48 M sodium phosphate, pH 6.8 (0.2 ml final volume) and incubated at 60°C for 2 hours. Following incubation, 5 ml of 2% hydroxyapatite, 0.12 M sodium phosphate pH 6.8, 0.02% sodium dodecyl sulfate was added and the mixture incubated at 60°C for 10 minutes. The sample was centrifuged and the supernatant removed. Five ml of wash solution (0.12 M sodium phosphate, pH 6.8, 0.02% sodium dodecyl sulfate) was added, the sample vortexed, centrifuged and the supernatant removed. The amount of radioactivity bound to the hydroxyapatite was determined by scintillation counting. The results are shown in Table 46 and demonstrates that the probe reacts well with E. cloacae and does not react with the RNA of closely related organisms.

TABLE 46

25 HYBRIDIZATION OF ENTEROBACTER CLOACAE PROBE  
TO CLOSELY RELATED ORGANISMS

Organisms Name	ATCC#	% Probe Bound
30 Citrobacter freundii	8090	1.8
Enterobacter aerogenes	13048	1.4
Enterobacter cloacae	13047	27.
Escherichia coli	11775	1.0
35 Klebsiella pneumoniae	13883	1.7
Proteus mirabilis	29906	0.9
Proteus vulgaris	13315	0.6
Providencia stuartii	29914	1.1

40

Table 47 shows that the probe does not react with the RNA of organisms found in urine.

45

50

55

TABLE 47

5

HYBRIDIZATION OF ENTEROBACTER CLOACAE  
PROBE TO ORGANISMS FOUND IN URINE.

	<u>Organisms Name</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
10	Candida albicans	18804	0.8
	Candida krusei	34135	0.8
	Candida parapsilosis	22019	0.9
	Candida tropicalis	750	1.1
15	Pseudomonas aeruginosa	10145	1.0
	Serratia marcescens	13880	1.6
	Staphylococcus aureus	12600	1.7
	Staphylococcus epidermidis	14990	1.4
	Streptococcus agalactiae	13813	2.5
20	Streptococcus faecium	19434	1.5
	Torulopsis glabrata	2001	0.9

Example 16

25

Members of the genus Proteus are motile, gram negative, non-sporeforming bacilli which belong in the family Enterobacteriaceae. Four species of Proteus have been described and three of them, Proteus mirabilis, P. vulgaris, and P. penneri, cause human disease.

30

The most common type of proteus infection involves the urinary tract, but septicemia, pneumonia and wound infections also occur. Proteus mirabilis is the species most often isolated and may account for up to 10% of all acute, uncomplicated urinary tract infections. Species, rather than genus level identification of the causative organism is desirable because of differential antibiotic susceptibility among the species.

35

The current method for identifying Proteus mirabilis from patient samples involves culture of the specimen on agar plates for 18-24 hours, followed by a battery of biochemical tests. The oligonucleotide sequence described below, when used as a probe in a nucleic acid hybridization assay, accurately identifies Proteus mirabilis. The present invention reduces the number of tests which must be run on a sample, the time to identification and therefore, diagnosis and treatment. This represents a significant improvement over prior art methods.

40

The probe specific for Proteus mirabilis was obtained with a primer complementary to a conserved region of 23S rRNA with the sequence 5'-CAG TCA GGA GTA TTT AGC CTT-3'.

The following sequence was characterized and shown to be specific for P. mirabilis. The phylogenetically nearest neighbors Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris and Salmonella enteritidis were used as comparisons with the sequence of Proteus mirabilis.

1. CCG TTC TCC TGA CAC TGC TAT TGA TTA AGA CTC

45

This probe is capable of hybridizing to the RNA of P. mirabilis in the region corresponding to base 270-305 of E. coli 23s rRNA. The probe is 33 bases in length and has a Tm of 66°C. To demonstrate the reactivity and specificity of the probe for P. mirabilis, it was used in a hybridization assay. <sup>32</sup>P-end-labeled oligonucleotide probe was mixed with RNA released from at least 10<sup>7</sup> organisms in 1% sodium dodecyl sulfate, 0.48 M sodium phosphate, pH 6.8, 1 mM EDTA, 1 mM EGTA (0.2 ml final volume) and incubated at 64°C for 2 hours. Following incubation, 5 ml of 2% hydroxyapatite, 0.12 M sodium phosphate pH 6.8, 0.02% sodium dodecyl sulfate was added and the mixture incubated at 64°C for 10 minutes. The sample was centrifuged and the supernatant removed. Five ml of wash solution (0.12 M sodium phosphate, pH 6.8, 0.02% sodium dodecyl sulfate) was added, the sample vortexed, centrifuged and the supernatant was removed. The amount of radioactivity bound to the hydroxyapatite was determined by scintillation counting.

50

The results are shown in Table 48 and demonstrate that the probe reacts well with P. mirabilis and does not react with 27 other closely related bacteria. Table 49 shows that the probe does not react with 24 other phylogenetically diverse bacteria and two yeasts tested in the same manner as the organisms in Table 48.

5

TABLE 48

HYBRIDIZATION OF PROTEUS MIRABILIS PROBE  
TO CLOSELY RELATED ORGANISMS

10

	<u>Organism Name</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
	Citrobacter diversus	27156	1.1
	Citrobacter freundii	8090	1.1
15	Citrobacter freundii	6750	1.0
	Enterobacter aerogenes	13048	1.0
	Enterobacter agglomerans	27155	1.0
	Enterobacter cloacae	813047	1.1
	Enterobacter gergoviae	33028	1.0
	Enterobacter sakazakii	29544	1.1
20	Escherichia coli	10798	1.2
	Escherichia coli	11775	1.2
	Escherichia coli	29417	1.2
	Klebsiella oxytoca	13182	1.0
	Klebsiella ozaenae	11296	1.1
25	Klebsiella planticola	33531	0.9
	Klebsiella pneumoniae	13883	1.3
	Klebsiella pneumoniae	23357	1.1
	Klebsiella rhinoscleromatis	13884	1.2
	Klebsiella terrigena	33257	1.1
	Klebsiella trevisanii	33558	1.0
30	Kluyvera ascorbata	33433	0.9
	Proteus mirabilis	25933	69.0
	Proteus penneri	33519	2.5
	Proteus vulgaris	13315	1.7
	Providencia alcalifaciens	9886	1.1
35	Providencia rettgeri	29944	1.3
	Providencia stuartii	29914	1.1
	Salmonella arizona	29933	1.1
	Salmonella enteritidis	13076	0.8

40

TABLE 49

HYBRIDIZATION OF PROTEUS MIRABILIS PROBE TO  
PHYLOGENETICALLY DIVERSE ORGANISMS

45

	<u>Organism Name</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
	Acinetobacter calcoaceticus	33604	0.8
	Bacillus subtilis	6051	1.2
50	Bacteroides fragilis	23745	0.9
	Branhamella catarrhalis	25238	0.7
	Campylobacter jejuni	33560	1.0
	Candida krusei	34135	0.8
	Chromobacterium violaceum	29094	1.1
	Clostridium perfringens	13124	0.9
55	Deinococcus radiodurans	35073	0.8

5

TABLE 48

HYBRIDIZATION OF PROTEUS MIRABILIS PROBE  
TO CLOSELY RELATED ORGANISMS

10

	<u>Organism Name</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
	Citrobacter diversus	27156	1.1
	Citrobacter freundii	8090	1.1
15	Citrobacter freundii	6750	1.0
	Enterobacter aerogenes	13048	1.0
	Enterobacter agglomerans	27155	1.0
	Enterobacter cloacae	813047	1.1
	Enterobacter gergoviae	33028	1.0
	Enterobacter sakazakii	29544	1.1
20	Escherichia coli	10798	1.2
	Escherichia coli	11775	1.2
	Escherichia coli	29417	1.2
	Klebsiella oxytoca	13182	1.0
	Klebsiella ozaenae	11296	1.1
25	Klebsiella planticola	33531	0.9
	Klebsiella pneumoniae	13883	1.3
	Klebsiella pneumoniae	23357	1.1
	Klebsiella rhinoscleromatis	13884	1.2
	Klebsiella terrigena	33257	1.1
	Klebsiella travisani	33558	1.0
30	Kluyvera ascorbata	33433	0.9
	Proteus mirabilis	25933	69.0
	Proteus penneri	33519	2.5
	Proteus vulgaris	13315	1.7
	Providencia alcalifaciens	9886	1.1
35	Providencia rettgeri	29944	1.3
	Providencia stuartii	29914	1.1
	Salmonella arizona	29933	1.1
	Salmonella enteritidis	13076	0.8

40

TABLE 49

HYBRIDIZATION OF PROTEUS MIRABILIS PROBE TO  
PHYLOGENETICALLY DIVERSE ORGANISMS

45

	<u>Organism Name</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
	Acinetobacter calcoaceticus	33604	0.8
	Bacillus subtilis	6051	1.2
50	Bacteroides fragilis	23745	0.9
	Branhamella catarrhalis	25238	0.7
	Campylobacter jejuni	33560	1.0
	Candida krusei	34135	0.8
	Chromobacterium violaceum	29094	1.1
	Clostridium perfringens	13124	0.9
55	Deinococcus radiodurans	35073	0.8

phosphate pH 6.8, 0.02% sodium dodecyl sulfate) was added and the samples were vortexed, centrifuged, and the supernatants removed. The amount of radioactivity bound to the hydroxyapatite was determined by scintillation counting. The results shown in Table 50 indicate that a combination of the two probes hybridized to the 5 subgroups of Salmonella and to all 31 of the serotypes which were tested.

5

10

15

TABLE 50

HYBRIDIZATION OF SALMONELLA PROBES 1 AND 2  
TO MEMBERS OF THE GENUS SALMONELLA

	<u>Subgroup</u>	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>	<u>Probe 1</u>	<u>Probe 2</u>
25	I	Salmonella choleraesuis	10708	24	40	
	I	Salmonella enteritidis	13076	15	67	
	I	Salmonella paratyphi A	9150	1.4	70	
	I	Salmonella sp. serotype anatum	9270	40	26	
30	I	Salmonella sp. serotype cubana	12007	54	35	
	I	Salmonella sp. serotype gizella	9268	12	40	
	I	Salmonella sp. serotype heidelberg	8326	53	33	
35	I	Salmonella sp. serotype illinois	11646	36	46	
	I	Salmonella sp. serotype montevideo	8387	35	32	
	I	Salmonella sp. serotype newington	29628	52	34	
40	I	Salmonella sp. serotype newport	6962	3.4	36	
	I	Salmonella sp. serotype puttan	15787	34	39	
	I	Salmonella sp. serotype saintpaul	9712	28	30	

45

50

55

	I	Salmonella sp. serotype sanftenberg	8400	38	43
5	I	Salmonella sp. serotype simsbury	12004	29	29
	I	Salmonella sp. serotype slotardijk	15791	34	30
	I	Salmonella sp. serotype thompson	8391	32	41
10	I	Salmonella sp. serotype vellore	15611	35	2.6
	I	Salmonella typhi	19430	7.0	21
	I	Salmonella typhimurium	14028	69	69
	II	Salmonella salamae	6959	3.0	46
	II	Salmonella sp. serotype maarssen	15793	6.6	30
15	III	Salmonella arizonae	33952	2.9	38
	III	Salmonella arizonae	12324	5.5	42
	III	Salmonella arizonae	29933	2.3	62
	III	Salmonella arizonae	29934	63	12
	III	Salmonella arizonae	12323	4.0	39
20	III	Salmonella arizonae	12325	51	1.9
	IV	Salmonella sp. serotype harmelen	15783	5.8	8.0
	IV	Salmonella sp. serotype ochsenzoll	29932	7.5	40
25	V	Salmonella sp. serotype bongor	cdc1319	60	1.8

The specificity of the probes for members of the genus Salmonella was demonstrated with hybridization reactions containing RNA from organisms closely related to Salmonella. The results are shown in Table 51.

TABLE 51

HYBRIDIZATION OF SALMONELLA PROBES 1 AND 2  
TO RNA OF CLOSELY RELATED ORGANISMS

Organism	ATCC#	% Probe Bound	
		Probe 1	Probe 2
Citrobacter freundii	6750	2.2	0
Edwardsiella tarda	15947	0	0
Enterobacter agglomerans	27155	0.6	0
Enterobacter cloacae	13047	0	0
Enterobacter sakazakii	29544	0	0
Escherichia coli	10798	0	0
Escherichia coli	29417	0	0
Klebsiella pneumoniae	23357	0.7	0
Kluyvera ascorbata	33433	0	0.5
Proteus mirabilis	25933	0.2	0
Shigella flexneri	29903	0	0

\*% Probe Bound = counts bound to hydroxyapatite - counts bound when no RNA present/total counts used in assay

Table 52 shows that *Salmonella* probes 1 and 2 do not hybridize to phylogenetically diverse organisms.

5

TABLE 52

HYBRIDIZATION OF SALMONELLA PROBES 1 AND 2 TO  
RNA OF A PHYLOGENETIC CROSS SECTION OF ORGANISMS

Organism	ATCC#	% Probe Bound*	
		Probe 1 and Probe 2	Probe 1
<i>Acinetobacter calcoaceticus</i>	33604	1.1	0.1
<i>Bacillus subtilis</i>	6051	0	0.5
<i>Bacteroides fragilis</i>	23745	0.1	0
<i>Branhamella catarrhalis</i>	25238	0.9	0
<i>Campylobacter jejuni</i>	33560	0	0.2
<i>Candida krusei</i>	34135	0.4	0.3
<i>Chromobacterium violaceum</i>	29094	1.7	0
<i>Clostridium perfringens</i>	13124	0.3	0
<i>Deinococcus radiodurans</i>	35073	1.6	0.1
<i>Darxia gummosa</i>	15994	1.2	0
<i>Hafnia alvei</i>	13337	1.8	0
<i>Morganella morganii</i>	25830	0	1.1
<i>Pseudomonas aeruginosa</i>	10145	0.5	0.7
<i>Pseudomonas cepacia</i>	17762	0	0
<i>Pseudomonas maltophilia</i>	13637	1.9	0
<i>Rahnella aquatilis</i>	33071	1.2	0.3
<i>Rhodospirillum rubrum</i>	11170	0.9	0
<i>Serratia marcescens</i>	13880	0	0
<i>Serratia odorifera</i>	33077	2.6	0.2
<i>Staphylococcus aureus</i>	612600	0.2	0
<i>Staphylococcus epidermidis</i>	14990	0	0
<i>Streptococcus mitis</i>	9811	1.2	0.7
<i>Streptococcus pneumoniae</i>	6306	0	0
<i>Torulopsis glabrata</i>	2001	0	0
<i>Vibrio parahaemolyticus</i>	17802	0	0.2
<i>Yersinia enterocolitica</i>	9610	0	0

\*% Probe Bound = Counts bound to hydroxyapatite - counts bound when no RNA present/total counts used in assay

40

Example 18

45

*Escherichia coli* is a gram negative, nonsporeforming bacillus which belongs in the family Enterobacteriaceae. Five species of *Escherichia* have been described: *E. coli*, which accounts for >99% of the clinical isolates, *E. hermanii*, *E. blattae*, *E. vulneris* and *E. fergusonii*. *E. coli* is a leading cause of urinary tract infections, bactemia and neonatal meningitis, and can cause a type of gastroenteritis known as traveller's diarrhea.

50

The current method for identifying *E. coli* from patient samples involves culture of the specimen on agar plates for 18-72 hours, followed by a battery of biochemical tests on isolated colonies. The oligonucleotide sequence described below, when used as a probe in a nucleic acid hybridization assay, accurately detects *E. coli* even in the presence of other organisms. The present invention reduces the number of tests which must be run on a sample and reduces the time to identification and therefore diagnosis and treatment. This represents a significant improvement over prior art methods.

The probe specific for *E. coli* was derived from the published *E. coli* sequence (Brosius, et al. Proc. Natl. Acad. Sci. U.S.A. 75:4801-4805 (1978)), using *Proteus vulgaris* (Carbon, et al., Nuc. Acids Res. 9:2325-2333

TABLE 53

5

## HYBRIDIZATION OF E. coli TO ESCHERICHIA SPECIES

	<u>Organism</u>	<u>ATCC#</u>	<u># Probe Bound</u>
10	Escherichia coli	10798	70
	E. coli	11775	67
	E. coli	23722	58
	E. coli	25404	68
	E. coli	25922	55
	E. coli	29417	72
15	E. coli	33780	0.8
	E. coli	35150	45
	E. fergusonii	35469	55
	E. hermanii	33650	0.7
	E. vulneris	33821	0.8

20

TABLE 54

## HYBRIDIZATION OF THE E. coli PROBE TO CLOSELY RELATED ORGANISMS

25

	<u>Organism</u>	<u>ATCC#</u>	<u># Probe Bound</u>
	Citrobacter freundii	6750	0.8
	Citrobacter freundii	8090	0.9
	Citrobacter freundii	29221	0.6
30	Citrobacter freundii	33128	0.6
	Enterobacter aerogenes	13048	1.2
	Enterobacter agglomerans	27155	0.9
	Enterobacter cloacae	13047	0.9
	Enterobacter gergoviae	33023	0.7
35	Enterobacter sakazakii	29544	0.6
	Klebsiella oxytoca	13182	0.7
	Klebsiella pneumoniae	13883	0.7
	Proteus mirabilis	29906	0.7
	Proteus vulgaris	13315	0.8
	Shibella boydii	8700	76
40	Shigella dysenteriae	13313	0.8
	Shigella flexneri	29903	71
	Shigella sonnei	29930	75

45

Example 19

The bacteria encompass a morphologically and physiologically diverse group of unicellular organisms which occupy most natural environments. Although many bacteria are harmless or beneficial to their environment or host, some are harmful and cause disease. The presence of any bacteria in some locations is undesirable or indicative of disease (e.g., culture media, pharmaceutical products, body fluids such as blood, urine or cerebrospinal fluid, and tissue biopsies). Low levels of bacteria are considered acceptable in other products such as drinking water and food products. Accordingly, there is a need for a means for detecting and quantitating bacteria in a sample.

The current method of detection and quantitation of total bacteria in a sample requires culture on multiple types of media under different conditions of temperature and atmosphere. To date, no single test exists to detect or quantitate all bacteria. The oligonucleotide sequences shown below, when used in a hybridization assay, detect a broad phylogenetic cross section of bacteria. The present invention reduces the number of tests which need to be performed and also reduces the time required for the assay.

TABLE 53

5

## HYBRIDIZATION OF E. coli TO ESCHERICHIA SPECIES

	<u>Organism</u>	<u>ATCC#</u>	<u># Probe Bound</u>
10	Escherichia coli	10798	70
	E. coli	11775	67
	E. coli	23722	58
	E. coli	25404	68
	E. coli	25922	55
	E. coli	29417	72
15	E. coli	33780	0.8
	E. coli	35150	45
	E. fergusonii	35469	55
	E. hermanii	33650	0.7
	E. vulneris	33821	0.8

20

TABLE 54

25

## HYBRIDIZATION OF THE E. coli PROBE TO CLOSELY RELATED ORGANISMS

	<u>Organism</u>	<u>ATCC#</u>	<u># Probe Bound</u>
	Citrobacter freundii	6750	0.8
	Citrobacter freundii	8090	0.9
	Citrobacter freundii	29221	0.6
30	Citrobacter freundii	33128	0.6
	Enterobacter aerogenes	13048	1.2
	Enterobacter agglomerans	27155	0.9
	Enterobacter cloacae	13047	0.9
	Enterobacter gergoviae	33023	0.7
35	Enterobacter sakazakii	29544	0.6
	Klebsiella oxytoca	13182	0.7
	Klebsiella pneumoniae	13883	0.7
	Proteus mirabilis	29906	0.7
	Proteus vulgaris	13315	0.8
	Shibella boydii	8700	76
40	Shigella dysenteriae	13313	0.8
	Shigella flexneri	29903	71
	Shigella sonnei	29930	75

45

Example 19

The bacteria encompass a morphologically and physiologically diverse group of unicellular organisms which occupy most natural environments. Although many bacteria are harmless or beneficial to their environment or host, some are harmful and cause disease. The presence of any bacteria in some locations is undesirable or indicative of disease (e.g., culture media, pharmaceutical products, body fluids such as blood, urine or cerebrospinal fluid, and tissue biopsies). Low levels of bacteria are considered acceptable in other products such as drinking water and food products. Accordingly, there is a need for a means for detecting and quantitating bacteria in a sample.

The current method of detection and quantitation of total bacteria in a sample requires culture on multiple types of media under different conditions of temperature and atmosphere. To date, no single test exists to detect or quantitate all bacteria. The oligonucleotide sequences shown below, when used in a hybridization assay, detect a broad phylogenetic cross section of bacteria. The present invention reduces the number of tests which need to be performed and also reduces the time required for the assay.

5

10

15

TABLE 55

HYBRIDIZATION OF BACTERIAL PROBE 1  
TO RNA OF ORGANISMS FOUND IN URINE

20

<u>Organism</u>	<u>ATCC‡</u>	<u>% Probe* Bound</u>
Candida albicans	18804	2.6
Candida krusei	34135	2.2
25 Candida parapsilosis	22019	2.9
Candida tropicalis	750	2.5
Citrobacter freundii	8090	69
Enterobacter aerogenes	13048	70
Enterobacter cloacae	13047	71
30 Escherichia coli	11775	67
Klebsiella oxytoca	13182	70
Klebsiella pneumoniae	13883	72
Morganella morganii	25830	66
Proteus mirabilis	29906	71
Proteus vulgaris	13315	67
35 Providencia stuartii	29914	69
Pseudomonas aeruginosa	10145	76
Pseudomonas fluorescens	13525	73
Serratia marcescens	13880	66
Staphylococcus aureus	12600	57
40 Staphylococcus epidermidis	14990	68
Streptococcus agalactiae	13813	68
Streptococcus faecalis	19433	51
Streptococcus faecium	19434	53
Torulopsis glabrata	2001	2.3
Ureaplasma urealyticum	27618	54

45

50

55

5

10

TABLE 56

15

HYBRIDIZATION OF BACTERIAL PROBE 1 TO RNAs  
OF A CROSS SECTION OF PHYLOGENETICALLY DIVERSE ORGANISMS.

		% Probe*	
	<u>Organism</u>	<u>ATCC#</u>	<u>Bound</u>
	<i>Acinetobacter calcoaceticus</i>	23055	65
25	<i>Bacillus subtilis</i>	6051	73
	<i>Bacteroides fragilis</i>	23745	61
	<i>Branhamella catarrhalis</i>	25238	72
30	<i>Campylobacter jejuni</i>	33560	64
	<i>Chlamydia trachomatis</i>	VR878	14
	<i>Chromabacterium violaceum</i>	29094	71
35	<i>Clostridium perfringens</i>	13124	74
	<i>Corynebacterium xerosis</i>	373	38
40	<i>Deinococcus radiodurans</i>	35073	47
	<i>Dexia gummosa</i>	15994	65
	<i>Gardnerella vaginalis</i>	14018	67
45	<i>Hafnia alvei</i>	13337	60
	<i>Lactobacillus acidophilus</i>	4356	56
	<i>Moraxalla osloensis</i>	19976	61
50	<i>Mycobacterium smegmatis</i>	14468	47
	<i>Mycoplasma hominis</i>	14027	58
	<i>Neisseria gonorrhoeae</i>	19424	58

55

<i>Rahnella aquatilis</i>	33071	74
<i>Rhodospirillum rubrum</i>	11170	73
<i>Vibrio parahaemolyticus</i>	17802	75
Human		2.5

10

Table 57 shows that Probe 2 hybridizes to the RNA of bacteria commonly found in urine except *Ureaplasma urealyticum* and does not hybridize to yeast rRNA.

15

TABLE 57

HYBRIDIZATION OF BACTERIAL PROBE 2  
TO RNA OF ORGANISMS FOUND IN URINE

20

<u>Organism</u>	<u>ATCC#</u>	<u>*Probe* Bound</u>
<i>Candida albicans</i>	18804	2.5
<i>Candida krusei</i>	34135	1.8
<i>Candida parapsilosis</i>	22019	1.6
<i>Candida tropicalis</i>	750	1.4
<i>Citrobacter freundii</i>	8090	61
<i>Enterobacter aerogenes</i>	13048	57
<i>Enterobacter cloacae</i>	13047	61
<i>Escherichia coli</i>	11775	67
<i>Klebsiella oxytoca</i>	13182	67
<i>Klebsiella pneumoniae</i>	13883	51
<i>Morganella morganii</i>	25830	69
<i>Proteus mirabilis</i>	29906	69
<i>Proteus vulgaris</i>	13315	69
<i>Providencia stuartii</i>	29914	66
<i>Pseudomonas aeruginosa</i>	10145	59
<i>Pseudomonas fluorescens</i>	13525	58
<i>Serratia marcescens</i>	13880	64
<i>Staphylococcus aureus</i>	12600	60
<i>Staphylococcus epidermidis</i>	14990	60
<i>Streptococcus agalactiae</i>	13813	54
<i>Streptococcus faecalis</i>	19433	37
<i>Streptococcus faecium</i>	19434	58
<i>Torulopsis glabrata</i>	2001	1.5
<i>Ureaplasma urealyticum</i>	27618	3.2

45

Table 58 shows that probe 2 detects phylogenetically diverse bacteria and does not hybridize to human rRNA.

50

55

TABLE 58

HYBRIDIZATION OF BACTERIAL PROBE 2 TO RNAs  
OF A CROSS SECTION OF PHYLOGENETICALLY DIVERSE ORGANISMS.

<u>Organism</u>	<u>ATCC#</u>	<u>% Probe* Bound</u>
Acinetobacter calcoaceticus	23055	76
Bacillus subtilis	6051	75
Bacteroides fragilis	23745	2.0
Branhamella catarrhalis	25238	70
Campylobacter jejuni	33560	2.5
Chlamydia trachomatis	VR878	16
Chromobacterium violaceum	29094	61
Clostridium perfringens	13124	66
Corynebacterium xerosis	373	3.8
Deinococcus radiodurans	35073	6.0
Derxia gummosa	15994	61
Gardnerella vaginalis	14018	2.0
Hafnia alvei	13337	72
Lactobacillus acidophilus	4356	50
Moraxalla osloensis	19976	64
Mycobacterium smegmatis	14468	19
Mycoplasma hominis	14027	34
Neisseria gonorrhoeae	19424	71
Rahnella aquatilis	33071	77
Rhodospirillum rubrum	11170	1.5
Vibrio parahaemolyticus	17802	73
Versinia enterocolitica	9610	76
Human		2.0

Table 59 shows that probe 3 hybridizes to the RNA of bacteria commonly found in urine and does not detect yeast rRNA.

TABLE 59

HYBRIDIZATION OF BACTERIAL PROBE 3 TO RNA OF  
ORGANISMS FOUND IN URINE.

<u>Organism</u>	<u>ATCC#</u>	<u>% Probe* Bound</u>
Candida albicans	18804	1.4
Candida krusei	34135	1.5
Candida parapsilosis	22019	2.2
Candida tropicalis	750	2.6
Citrobacter freundii	8090	79

	Enterobacter aerogenes	13048	40
5	Enterobacter cloacae	13047	44
	Escherichia coli	11775	67
	Klebsiella oxytoca	13182	38
	Klebsiella pneumoniae	13883	45
	Morganella morganii	25830	57
	Proteus mirabilis	29906	40
	Proteus vulgaris	13315	51
10	Providencia stuartii	29914	54
	Pseudomonas aeruginosa	10145	61
	Pseudomonas fluorescens	13525	56
	Serratia marcescens	13880	54
	Staphylococcus aureus	12600	37
	Staphylococcus epidermidis	14990	20
15	Streptococcus agalactiae	13813	34
	Streptococcus faecalis	19433	20
	Streptococcus faecium	19434	47
	Torulopsis glabrata	2001	1.9
	Ureaplasma urealyticum	27618	26

20 Table 60 shows that probe 3 detects phylogenetically diverse bacteria and does not hybridize to human rRNA.

25

TABLE 60

HYBRIDIZATION OF BACTERIAL PROBE 3 TO RNAs  
OF A CROSS SECTION OF PHYLOGENETICALLY DIVERSE ORGANISMS.

	<u>Organism Name</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
	Acinetobacter calcoaceticus	23055	69
	Bacillus subtilis	6051	35
36	Bacteroides fragilis	23745	1.2
	Branhamella catarrhalis	25238	43
	Campylobacter jejuni	33560	55
	Chlamydia trachomatis	VR878	42
	Chromobacterium violaceum	29094	69
	Clostridium perfringens	13124	62
40	Corynebacterium xerosis	373	23
	Deinococcus radiodurans	35073	30
	Derxia gummosa	15994	67
	Gardnerella vaginalis	14018	40
	Hafnia alvei	13337	56
45	Lactobacillus acidophilus	4356	36
	Moraxella osloensis	19976	64
	Mycobacterium smegmatis	14468	77
	Mycoplasma hominis	14027	1.5
	Neisseria gonorrhoeae	19424	26
	Rahnella aquatilis	33071	66
50	Rhodospirillum rubrum	11170	51
	Vibrio parahaemolyticus	17802	68
	Yersinia enterocolitica	9610	68
	Human		0.9

55 Table 61 shows that probe 4 hybridizes to the RNA of bacteria commonly found in urine and does not detect yeast rRNA.

TABLE 61

5 HYBRIDIZATION OF BACTERIAL PROBE 4 TO RNA OF  
ORGANISMS FOUND IN URINE.

<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
10 <i>Candida albicans</i>	18804	4.5
<i>Candida krusei</i>	34135	2.5
<i>Candida parapsilosis</i>	22019	2.7
<i>Candida tropicalis</i>	750	2.5
15 <i>Citrobacter freundii</i>	8090	55
<i>Enterobacter aerogenes</i>	13048	52
15 <i>Enterobacter cloacae</i>	13047	57
<i>Escherichia coli</i>	11775	70
<i>Klebsiella oxytoca</i>	13182	70
<i>Klebsiella pneumoniae</i>	13883	43
20 <i>Morganella morganii</i>	25830	74
<i>Proteus mirabilis</i>	29906	74
<i>Proteus vulgaris</i>	13315	73
<i>Providencia stuartii</i>	29914	73
<i>Pseudomonas aeruginosa</i>	10145	76
<i>Pseudomonas fluorescens</i>	13525	79
25 <i>Serratia marcescens</i>	13880	74
<i>Staphylococcus aureus</i>	12600	73
<i>Staphylococcus epidermidis</i>	14990	73
<i>Streptococcus agalactiae</i>	13813	70
25 <i>Streptococcus faecalis</i>	19433	37
30 <i>Streptococcus faecium</i>	19434	63
<i>Torulopsis glabrata</i>	2001	2.2
<i>Ureaplasma urealyticum</i>	27618	43

35 Table 62 shows that probe 4 detects phylogenetically diverse bacteria and does not hybridize to human rRNA.

40

TABLE 63

45 HYBRIDIZATION OF BACTERIAL PROBE 4 TO RNAs  
OF A CROSS SECTION OF PHYLOGENETICALLY DIVERSE  
ORGANISMS

50

% Probe

55

	<u>Organism Name</u>	<u>ATCC#</u>	<u>Bound</u>
5	<i>Acinetobacter calcoaceticus</i>	23055	69
	<i>Bacillus subtilis</i>	6051	55
	<i>Bacteroides fragilis</i>	23745	3.0
	<i>Branhamella catarrhalis</i>	25238	59
	<i>Campylobacter jejuni</i>	33560	65
	<i>Chlamydia trachomatis</i>	VR878	50
10	<i>Chromobacterium violaceum</i>	29094	61
	<i>Clostridium perfringens</i>	13124	57
	<i>Corynebacterium xerosis</i>	373	9.5
	<i>Deinococcus radiodurans</i>	35073	63
	<i>Dexia gummosa</i>	15994	65
15	<i>Gardnerella vaginalis</i>	14018	57
	<i>Hafnia alvei</i>	13337	67
	<i>Lactobacillus acidophilus</i>	4356	68
	<i>Moraxella osloensis</i>	19976	68
	<i>Mycobacterium smegmatis</i>	14468	28
	<i>Mycoplasma hominis</i>	14027	74
20	<i>Neisseria gonorrhoeae</i>	19424	76
	<i>Rahnella aquatilis</i>	33071	68
	<i>Rhodospirillum rubrum</i>	11170	59
	<i>Vibrio parahaemolyticus</i>	17802	75
	<i>Yersinia enterocolitica</i>	9610	74
25	Human		2.8

Table 63 shows that probe 5 hybridizes to the RNA of bacteria commonly found in urine and does not detect yeast rRNA.

30

35

40

45

50

55

TABLE 63

5 HYBRIDIZATION OF BACTERIAL PROBE 5 TO RNA OF  
ORGANISMS FOUND IN URINE.

<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
Candida albicans	18804	1.8
Candida krusei	34135	1.7
Candida parapsilosis	22019	2.2
Candida tropicalis	750	1.8
Citrobacter freundii	8090	39
Enterobacter aerogenes	13048	38
Enterobacter cloacae	13047	43
Escherichia coli	11775	31
Klebsiella oxytoca	13182	38
Klebsiella pneumoniae	13883	66
Morganella morganii	25830	50
Proteus mirabilis	29906	44
Proteus vulgaris	13315	52
Providencia stuartii	29914	44
Pseudomonas aeruginosa	10145	47
Pseudomonas fluorescens	13525	25
Serratia marcescens	13880	35
Staphylococcus aureus	12600	26
Staphylococcus epidermidis	14990	37
Streptococcus agalactiae	13813	29
Streptococcus faecalis	19433	14
Streptococcus faecium	19434	33
Torulopsis glabrata	2001	2.2
Ureaplasma urealyticum	27618	73

40 Table 64 shows that probe 5 detects phylogenetically diverse bacteria and does not hybridize to human  
35 RNA.

TABLE 64

40 HYBRIDIZATION OF BACTERIAL PROBE 5 TO RNAs  
OF A CROSS SECTION OF PHYLOGENETICALLY DIVERSE  
ORGANISMS

<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
Acinetobacter calcoaceticus	23055	20
Bacillus subtilis	6051	53
Bacteroides fragilis	23745	44
Branhamella catarrhalis	25238	22
Campylobacter jejuni	33560	35
Chromabacterium violaceum	29094	59
Clostridium perfringens	13124	63

55

	<i>Corynebacterium xerosis</i>	373	1.7
5	<i>Deinococcus radiodurans</i>	35073	5.7
	<i>Derxia gummosa</i>	15994	14
	<i>Gardnerella vaginalis</i>	14018	1.6
	<i>Hafnia alvei</i>	13337	44
	<i>Lactobacillus acidophilus</i>	4356	1.5
	<i>Moraxella osloensis</i>	19976	7.3
	<i>Mycobacterium smegmatis</i>	14468	39
10	<i>Mycoplasma hominis</i>	14027	21
	<i>Neisseria gonorrhoeae</i>	19424	40
	<i>Rahnella aquatilis</i>	33071	55
	<i>Rhodospirillum rubrum</i>	11170	17
	<i>Vibrio parahaemolyticus</i>	17802	66
15	<i>Yersinia enterocolitica</i>	9510	64
	<b>Human</b>		1.6

Table 65 shows that probe 6 hybridizes to the RNA of bacteria commonly found in urine and does not detect yeast rRNA.

20

TABLE 65

**HYBRIDIZATION OF BACTERIAL PROBE 6 TO RNA OF  
ORGANISMS FOUND IN URINE**

25

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
	<i>Candida albicans</i>	18804	3.0
30	<i>Candida krusei</i>	34135	2.0
	<i>Candida parapsilosis</i>	22019	2.2
	<i>Citrobacter freundii</i>	8090	54
	<i>Enterobacter aerogenes</i>	13048	50
	<i>Enterobacter cloacae</i>	13047	58
	<i>Escherichia coli</i>	11775	63
35	<i>Klebsiella oxytoca</i>	13182	54
	<i>Klebsiella pneumoniae</i>	13883	55
	<i>Morganella morgani</i>	25830	60
	<i>Proteus mirabilis</i>	29906	64
	<i>Proteus vulgaris</i>	13315	67
40	<i>Providencia stuartii</i>	29914	64
	<i>Pseudomonas aeruginosa</i>	10145	65
	<i>Pseudomonas fluorescens</i>	13525	31
	<i>Serratia marcescens</i>	13880	67
	<i>Staphylococcus aureus</i>	12600	53
	<i>Staphylococcus epidermidis</i>	14990	34
45	<i>Streptococcus agalactiae</i>	13813	31
	<i>Streptococcus faecium</i>	19434	18
	<i>Torulopsis glabrata</i>	2001	2.5

50

Table 66 shows that probe 6 detects some phylogenetically diverse bacteria and does not hybridize to human rRNA.

55

TABLE 66

HYBRIDIZATION OF BACTERIAL PROBE 5 TO RNAs  
OF A CROSS SECTION OF PHYLOGENETICALLY  
DIVERSE ORGANISMS.

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
	Acinetobacter calcoaceticus	23055	73
	Bacteroides fragilis	23745	7.0
	Branhamella catarrhalis	25238	4.0
15	Deinococcus radiodurans	35073	5.5
	Derxia gummosa	15994	3.0
	Gardnarella vaginalis	14018	2.0
	Hafnia alvei	13337	3.5
	Lactobacillus acidophilus	4356	17
20	Moraxella osloensis	19976	62
	Mycoplasma hominis	14027	44
	Rahnella aquatilis	33071	56
	Versinia enterocolitica	9610	50
	Human		4.0

25 Table 67 shows that probe 7 hybridizes to the RNA of bacteria commonly found in urine and does not detect yeast rRNA.

TABLE 67

HYBRIDIZATION OF BACTERIAL PROBE 7 TO RNA  
OF ORGANISMS FOUND IN URINE

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
	Candida albicans	18804	2.1
	Candida krusei	34135	2.0
	Candida tropicalis	750	2.2
45	Citrobacter freundii	8090	67
	Enterobacter aerogenes	13048	69
	Enterobacter cloacae	13047	78
	Escherichia coli	11775	75
	Klebsiella oxytoca	13882	79
	Klebsiella pneumoniae	13883	77
50	Morganella morganii	25830	76
	Proteus mirabilis	29906	77

	Proteus vulgaris	13315	79
5	Providencia stuartii	29914	64
	Pseudomonas aeruginosa	10145	76
	Pseudomonas fluorescens	13525	78
	Serratia marcescens	13880	66
	Staphylococcus aureus	12600	71
	Staphylococcus epidermidis	14990	75
	Streptococcus agalactiae	13813	70
10	Streptococcus faecalis	19433	58
	Streptococcus faecium	19434	68
	Torulopsis glabrata	2001	2.4
	Ureaplasma urealyticum	27618	21

15 Table 68 shows that probe 7 detects phylogenetically diverse bacteria and does not hybridize to human rRNA.

20

TABLE 68

HYBRIDIZATION OF BACTERIAL PROBE 7 TO RNAs  
OF A CROSS SECTION OF PHYLOGENETICALLY  
DIVERSE ORGANISMS

25

	<u>Organism</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
	Acinetobacter calcoaceticus	23055	86
30	Bacillus subtilis	6051	83
	Bacteroides fragilis	23745	69
	Branhamella catarrhalis	25238	74
	Campylobacter jejuni	33560	5.3
	Chlamydia trachomatis	VR878	41
	Chromobacterium violaceum	29094	69
35	Clostridium perfringens	13124	68
	Corynebacterium xerosis	373	23
	Deinococcus radiodurans	35073	70
	Dexia gummosa	15994	69
	Gardnerella vaginalis	14018	68
	Hafnia alvei	13337	77
40	Moraxella osloensis	19976	68
	Mycobacterium smegmatis	14468	64
	Mycoplasma hominis	14027	4.0
	Neisseria gonorrhoeae	19424	53
	Rahnella aquatilis	33071	72
45	Rhodospirillum rubrum	11170	73
	Vibrio parahaemolyticus	17802	67
	Yersinia enterocolitica	9610	66
	Human		2.2

50

Example 20

Fungi encompass a morphologically and physiologically diverse group of simple eucaryotic organisms. We estimate, using published sequences of three fungi, Neurospora crassa, Podospora, and Saccharomyces, that the rRNA of fungi are 58-60% homologous to E. coli and 84-90% homologous to one another. Some fungi grow as single cells (yeasts), others as multinuclear filaments (molds) and still others can grow as either single cells or multicellular filaments (dimorphic fungi). Although many fungi are

	Proteus vulgaris	13315	79
5	Providencia stuartii	29914	64
	Pseudomonas aeruginosa	10145	76
	Pseudomonas fluorescens	13525	78
	Serratia marcescens	13880	66
	Staphylococcus aureus	12600	71
	Staphylococcus epidermidis	14990	75
	Streptococcus agalactiae	13813	70
10	Streptococcus faecalis	19433	58
	Streptococcus faecium	19434	68
	Torulopsis glabrata	2001	2.4
	Ureaplasma urealyticum	27618	21

15 Table 68 shows that probe 7 detects phylogenetically diverse bacteria and does not hybridize to human rRNA.

20

TABLE 68

HYBRIDIZATION OF BACTERIAL PROBE 7 TO RNAs  
OF A CROSS SECTION OF PHYLOGENETICALLY  
DIVERSE ORGANISMS

25

	<u>Organism</u>	<u>ATCC#</u>	<u>t Probe Bound</u>
	Acinetobacter calcoaceticus	23055	86
30	Bacillus subtilis	6051	83
	Bacteroides fragilis	23745	69
	Branhamella catarrhalis	25238	74
	Campylobacter jejuni	33560	5.3
	Chlamydia trachomatis	VR878	41
	Chromobacterium violaceum	29094	69
35	Clostridium perfringens	13124	68
	Corynebacterium xerosis	373	23
	Deinococcus radiodurans	35073	70
	Dexia gummosa	15994	69
	Gardnerella vaginalis	14018	68
	Hafnia alvei	13337	77
40	Moraxella osloensis	19976	68
	Mycobacterium smegmatis	14468	64
	Mycoplasma hominis	14027	4.0
	Neisseria gonorrhoeae	19424	53
	Rahnella aquatilis	33071	72
45	Rhodospirillum rubrum	11170	73
	Vibrio parahaemolyticus	17802	67
	Yersinia enterocolitica	9610	66
	Human		2.2

50

Example 20

Fungi encompass a morphologically and physiologically diverse group of simple eucaryotic organisms. 55 We estimate, using published sequences of three fungi, Neurospora crassa, Podospora, and Saccharomyces, that the rRNA of fungi are 58-80% homologous to E. coli and 84-90% homologous to one another. Some fungi grow as single cells (yeasts), others as multinuclear filaments (molds) and still others can grow as either single cells or multicellular filaments (dimorphic fungi). Although many fungi are

5

TABLE 69

## HYBRIDIZATION OF YEAST PROBES TO YEAST RNA

<u>Organism</u>	ATCC#	% Probe Bound			
		#1	#2	#3	#4
<i>Blastomyces dermatitidis</i>	C.I.	25	1.4	1.5	1.5
<i>Candida albicans</i>	18804	40	63	56	2.0
<i>C. krusei</i>	34135	73	62	2.2	70
<i>C. parapsilosis</i>	22019	71	63	65	2.0
<i>C. tropicalis</i>	750	62	71	71	2.0
<i>Cryptococcus laurentii</i>	C.I.	43	1.4	1.5	1.5
<i>Cryptococcus neoformans</i>	C.I.	60	1.3	1.5	1.6
<i>Torulopsis glabrata</i>	2001	61	44	62	2.0
<i>Trichosporon beigelii</i>	C.I.	57	1.3	2.1	1.5
<i>Saccharomyces cerevisiae</i>	C.I.	41	67	53	1.9

C.I. = Clinical isolate

25

TABLE 70

HYBRIDIZATION OF FUNGAL PROBES 1-4 TO RNA  
OF ORGANISMS FOUND IN URINE

<u>Organism</u>	ATCC#	% Probe Bound			
		#1	#2	#3	#4
<i>Citrobacter freundii</i>	8090	1.5	1.7	1.5	2.1
<i>Enterobacter aerogenes</i>	13048	2.5	1.9	2.0	2.0
<i>Enterobacter cloacae</i>	13047	2.5	1.6	2.6	2.0
<i>Escherichia coli</i>	11775	3.0	2.0	1.6	1.5
<i>Klebsiella oxytoca</i>	13182	2.5	2.2	2.5	2.0
<i>Klebsiella pneumoniae</i>	13883	2.5	2.2	2.1	2.0
<i>Morganella morganii</i>	25830	2.0	2.8	1.7	1.9
<i>Proteus mirabilis</i>	29906	2.5	1.9	2.3	2.0
<i>Proteus vulgaris</i>	13315	2.0	2.2	2.0	1.5
<i>Providencia stuartii</i>	29914	3.0	1.7	2.8	2.0
<i>Pseudomonas aeruginosa</i>	10145	2.0	1.9	1.3	2.0
<i>Pseudomonas fluorescens</i>	13525	2.5	2.7	2.1	2.0
<i>Serratia marcescens</i>	13880	2.5	1.7	1.8	2.0
<i>Staphylococcus aureus</i>	12600	2.0	1.7	1.8	2.0
<i>Staphylococcus epidermidis</i>	14990	3.0	1.5	1.3	2.0
<i>Streptococcus agalactiae</i>	13813	2.5	1.9	1.3	2.5
<i>Streptococcus faecalis</i>	19433	1.7	3.3	3.5	1.9
<i>Streptococcus faecium</i>	19434	2.0	2.9	2.1	1.5
<i>Ureaplasma urealyticum</i>	27618	2.1	3.1	2.4	1.8

55

greater DNA:DNA homology. Despite the fact that these organisms may be considered to be the same species under established principles, we were able to make probes capable of distinguishing them.

As expected, the rRNA homology between N. gonorrhoeae and N. meningitidis is even greater because of known conserved regions. We noted a 1.0% difference between the 18S and a 1.1% difference between 5 the 23S rRNA sequences of N. gonorrhoeae and N. meningitidis using our sequencing data.

Making a probe for N. gonorrhoeae was complicated by the fact that in some sites where N. meningitidis and N. gonorrhoeae differed, other Neisseria species were similar to N. gonorrhoeae. The few mismatches which exist between these two species are in the most variable regions, i.e., regions which vary not only between species, but also from strain to strain. Despite the fact that some believed the species 10 could not be distinguished at all, and others believed that rRNA was too conserved to be useful in probe diagnostics, we were able to make probes capable of differentiating N. gonorrhoeae and N. meningitidis.

The present invention has significant advantages over each of the prior art methods; the probes are more specific and much faster than culture methods. It also is believed that the probes are more sensitive, (i.e., able to detect a smaller number of organisms in a clinical sample) than prior art methods.

15 The primers used to identify these probe sequences had the following sequences:

1. GGCGT TACCC CACCT ACTAG CTAAT
2. GTATTACCGCGGCTGCTGGCAC

3. GCTCGTTGC GGGACTTA ACCC ACCAT Each of the rRNA sites chosen to target had at least two mismatches to E. coli, N. meningitidis, N. cinerea, N. lactamica, N. mucosa, and Kingella kingae.

20 Oligonucleotides complementary to sequences adjacent to the probe regions were synthesized and used in the hybridization mix according to Hogan et al., U.S. Patent App. Ser. No. (not yet assigned), entitled "Means and Method for enhancing nucleic acid hybridisation".

The following sequences were characterized and shown to be specific for Neisseria gonorrhoeae. The phylogenetically nearest neighbors Neisseria meningitidis, N. lactamica, N. cinerea, N. mucosa, and 25 Kingella kingae were used for comparison with the N. gonorrhoeae sequence.

1. CCG CCG CTA CCC GGT AC  
 2. TCA TCG GCC GCC GAT ATT GGC  
 3. GAG CAT TCC GCA CAT GTC AAA ACC AGC AGG TA Sequence 1, complementary to 18S rRNA in the region 125-150, is 17 bases in length and has a Tm of 56°C. Sequence 2, complementary to 18S rRNA 30 in the region 455-485, is 21 bases in length and has a Tm of 63°C. Sequence 3, complementary to 18S rRNA in the region 980-1015, is 29 bases in length and has a Tm of 57°C.

The reactivity and specificity of the probes for Neisseria gonorrhoeae was demonstrated with a hybridization assay. The three oligonucleotide probes were iodinated and mixed with unlabeled oligonucleotides of sequence 5'-CCC CTG CTT TCC CTC TCT AGA CGT ATG CGG TAT TAG CTG ATC 35 TTT CG-3', 5'-GCC TTT TCT TCC CTG ACA AAA GTC CTT TAC AAC CCG-3', 5'-GGC ACG TAG TTA GCC GGT GCT TAT TCT TCA GGT AC-3', and 5'-GGT TCT TCG CGT TGC ATC GAA TTA ATC CAC ATC ATC CAC CGC-3', and with purified RNA in 0.48 M sodium phosphate, pH 8.8, 0.5% sodium dodecyl sulfate (SDS) and incubated at 60°C for one hour. Following incubation, 4 ml of 2% hydroxyapatite, 0.12 M sodium phosphate pH 8.8, 0.02% SDS was added and the mixture was incubated at 60°C for 5 minutes. The 40 samples were centrifuged and the supernatants were removed. Five ml of wash solution (0.12 M sodium phosphate pH 8.8, 2% SDS) was added and the samples were mixed, centrifuged, and the supernatants removed. The amount of radioactivity bound to the hydroxyapatite was determined in a gamma counter.

Table 72 shows that the probes hybridize well to N. gonorrhoeae RNA and do not hybridize to the other species tested.

45

50

55

TABLE 72

5            HYBRIDIZATION OF NEISSERIA GONORRHOEAE  
           PROBES 1-3 TO NEISSERIA AND KINGELLA RNAs

	<u>Organisms</u>	<u>ATCC#</u>	<u>% Probe Bound</u>
10	<u>Kingella kingae</u>	23332	0.09
	<u>Neisseria cinerea</u>	14685	0.04
	<u>N. gonorrhoeae</u>	19424	48.4
	<u>N. lactamica</u>	23970	0.07
	<u>N. meningitidis</u> serogroup A	13077	0.04
15	<u>N. meningitidis</u> serogroup B	13090	0.04
	<u>N. meningitidis</u> serogroup C	13102	0.04
	<u>N. mucosa</u>	19696	0.07
	<u>N. subflava</u>	14799	0.05

20         The following derivatives of Neisseria probes also have been made and used:

GAG GAT TCC GCA CAT GTC AAA ACC AGG  
   GAG GAT TCC GCA CAT GTC AAA ACC AGG TAA  
   CCC GCT ACC CGG TAC GTT C  
   CCG CTA CCC GGT ACG TTC.

25         Although the above examples of performance were determined using the standard assay format previously described, the specific probes may be used under a wide variety of experimental conditions. For example, additives may be included to the reaction solutions to provide optimal reaction conditions for accelerated hybridization. Such additives may include buffers, chelators, organic compounds and nucleic acid precipitating agents such as detergents, dihydroxybenzene, sodium dodecyl sulfate, sodium dibutyl sulfosuccinate, sodium tetradecyl sulfate, sarkosyl and the alkali metal salts and ammonium salts of SO-2, PO-3, Cl- and HCOO-. Such additives can be utilized by one skilled in the art to provide optimal conditions for the hybridization reaction to take place. These conditions for accelerated hybridization of single stranded nucleic acid molecules into double stranded molecules are the subject of the above-noted U.S. Patent App. Ser. No. 627,795 filed July 5, 1984, continuation filed June 4, 1987 (serial no. not yet assigned) (EP-A-0167388) and Ser. No. 816,711 filed January 7, 1986 (EP-A-0229442) which are both entitled ACCELERATED NUCLEIC ACID REASSOCIATION METHOD.

30         The present invention can be carried out on nonviral organisms from purified samples or unpurified clinical samples such as sputum, feces, tissue, blood, spinal or synovial fluids serum, urine or other bodily fluids, or other samples such as environmental or food samples. Prior to cell breakage and hybridization, the cells can be suspended or placed in solution. In the case of the unpurified samples referred to above, the cells may remain intact and untreated in their own biological environment prior to the assay.

35         The probes of the present invention may be used in an assay either alone or in combination with different probes. Several individual probes also can be linked together during nucleic acid synthesis. This results in one probe molecule which contains multiple probe sequences, and therefore, multiple specificities. For example, a single nucleic acid molecule can be synthesized which contains both the Mycobacterium avium and the Mycobacterium intracellulare sequences described in Examples 1 and 2. When hybridized with either M. avium or M. intracellulare rRNA this probe will hybridize completely. If the two probe sequences were combined separately in an assay only one half of the mixed individual probes will hybridize with either M. avium or M. intracellulare rRNA. Other embodiments also may be practiced within the scope of the claims. For example, probes may be labelled using a variety of labels, as described within, and may be incorporated into diagnostic kits.

#### Claims

40         1. A method for preparing a probe for use in a qualitative or quantitative hybridization assay which comprises constructing an oligonucleotide that is sufficiently complementary to hybridize to a region of rRNA selected to be unique to a non-viral organism or group of non-viral organisms sought to be detected.

- said region of rRNA being selected by comparing one or more variable region rRNA sequences of said non-viral organism or group of non-viral organisms with one or more variable region rRNA sequences from one or more non-viral organisms sought to be distinguished therefrom.
2. The method of claim 1 wherein said variable region rRNA sequences from non-viral organisms sought to be distinguished are from the known nearest related organism to said non-viral organism or group of non-viral organisms sought to be detected.
3. The method of claim 1 wherein said region of rRNA is selected to have at least about a one base sequence difference from a corresponding rRNA sequence of the known nearest related organism to said non-viral organism or group of non-viral organisms sought to be detected.
- 10 4. The method of claim 1 wherein said region of rRNA is selected to have at least about a 10% or greater base sequence difference from the corresponding rRNA sequence of the known nearest related organism to said non-viral organism or group of non-viral organisms sought to be detected.
5. The method of claim 1 wherein said region of rRNA is chosen from the group consisting of 5S, 16S, and 23S rRNA.
- 15 6. The method of claim 1 wherein said region of rRNA is chosen from the group consisting of 5.0S, 5.8S, 18S and 28S rRNA.
7. The method of claim 1 wherein said oligonucleotide is at least about 10 nucleotides in length.
8. The method of claim 1 wherein said oligonucleotide is at least about 15 nucleotides in length.
9. The method of claim 1 wherein said oligonucleotide is at least about 20 nucleotides in length.
- 20 10. The method of claim 1 wherein said oligonucleotide is at least about 30 nucleotides in length.
11. The method of claim 1 wherein said oligonucleotide is about 20 nucleotides to about 50 nucleotides in length.
12. The method of claim 1 wherein said oligonucleotide is about 30 nucleotides to about 50 nucleotides in length.
- 25 13. The method of claim 3 wherein said oligonucleotide is at least about 10 nucleotides in length.
14. The method of claim 3 wherein said oligonucleotide is at least about 15 nucleotides in length.
15. The method of claim 1 wherein said oligonucleotide is at least about 20 nucleotides in length.
16. The method of claim 3 wherein said oligonucleotide is at least about 30 nucleotides in length.
- 30 17. The method of claim 3 wherein said oligonucleotide is about 20 nucleotides to about 50 nucleotides in length.
18. The method of claim 3 wherein said oligonucleotide is at about 30 nucleotides to about 50 nucleotides in length.
19. The method of claim 4 wherein said oligonucleotide is at least about 10 nucleotides in length.
20. The method of claim 4 wherein said oligonucleotide is at least about 15 nucleotides in length.
- 35 21. The method of claim 4 wherein said oligonucleotide is at least about 20 nucleotides in length.
22. The method of claim 4 wherein said oligonucleotide is at least about 30 nucleotides in length.
23. The method of claim 4 wherein said oligonucleotide is about 20 nucleotides to about 50 nucleotides in length.
24. The method of claim 4 wherein said oligonucleotide is about 30 nucleotides to about 50 nucleotides in length.
- 40 25. The method of claim 1 wherein said probe is at least about 75% complementary to said region of rRNA.
26. The method of claim 3 wherein said oligonucleotide is at least about 75% complementary to said region of rRNA.
- 45 27. The method of claim 4 wherein said oligonucleotide is at least about 75% complementary to said region of rRNA.
28. The method of claim 1 wherein said probe is perfectly complementary to said region of rRNA.
29. The method of claim 3 wherein said probe is perfectly complementary to said region of rRNA.
30. The method of claim 4 wherein said probe is perfectly complementary to said region of rRNA.
- 50 31. A hybridization assay probe for a non-viral organism or organisms comprising an oligonucleotide of at least about 10 nucleotides in length wherein at least about 10 contiguous nucleotides are substantially complementary to at least one variable region of nucleic acid selected to be unique to said non-viral organism or organisms.
32. A hybridization assay probe for a non-viral organism or organisms comprising an oligonucleotide of at least about 10 nucleotides in length which is at least about 75% complementary to at least one variable region of nucleic acid selected to be unique to said non-viral organism or organisms.
- 55 33. The probe of claim 31 or 32 wherein said nucleic acid is 5S, 18S, or 23S rRNA.
34. The probe of claim 31 or 32 wherein said nucleic acid is 5.0S, 5.8S, 18S, or 28S rRNA.

35. The probe of claim 31 wherein said non-viral organism is Mycobacterium avium.
36. The probe of claim 35 wherein said oligonucleotide comprises the sequence ACCGCAAAAGCTT-TCCACCAAGAACATGCGTCTTGAG.
37. A nucleotide polymer capable of hybridizing to the probe of claim 36 or to the complement thereof.
- 5 38. A nucleic acid hybrid formed between an oligonucleotide comprising the sequence  
ACCGCAAAAGCTTCCACCAAGAACATGCGTCTTGAG and a nucleic acid sequence substantially complementary thereto.
39. A nucleotide polymer of the structure ACCGCAAAAGCTTCCACCAAGAACATGCGTCTTGAG and the complement thereto.
- 10 40. A nucleotide polymer capable of hybridizing to RNA of the species Mycobacterium avium in the region corresponding to bases 185-225 of E. coli 16S rRNA.
41. A nucleic acid hybrid formed between a nucleotide polymer of claim 40 and a nucleic acid sequence substantially complementary thereto.
- 15 42. The probe of claim 31 wherein said non-viral organism is Mycobacterium intracellulare.
43. The probe of claim 42 wherein said oligonucleotide comprises the sequence  
ACCGCAAAAGCTTCCACCTAAAGAACATGCGCCTAAAG.
44. A nucleotide polymer capable of hybridizing to the probe of claim 43 or to the complement thereof.
- 20 45. A nucleic acid hybrid formed between an oligonucleotide comprising the sequence  
ACCGCAAAAGCTTCCACCTAAAGAACATGCGCCTAAAG and a nucleic acid sequence substantially complementary thereto.
46. A nucleotide polymer of the structure ACCGCAAAAGCTTCCACCTAAAGAACATGCGCCTAAAG and the complement thereto.
47. A nucleotide polymer capable of hybridizing to the RNA of the species Mycobacterium intracellulare in the region corresponding to bases 185-225 of E. coli 16S rRNA.
- 25 48. A nucleic acid hybrid formed between a nucleotide polymer of claim 47 and a nucleic acid sequence substantially complementary thereto.
49. The probe of claim 31 wherein said non-viral organisms are the Mycobacterium tuberculosis-complex bacteria.
50. The probe of claim 49 wherein said oligonucleotide comprises the sequence TAAAGCGCTTCCAC-
- 30 CACAAGAACATGCATCCCGTG.
51. The probe of claim 49 wherein said oligonucleotide comprises the sequence TGCCCTACCCACAC-CCACCACGAGGTGATGT.
52. The probe of claim 49 wherein said oligonucleotide comprises the sequence CCATCACCAACCCCTC-CTCCGGAGAGGAAAAGG.
- 35 53. The probe of claim 49 wherein said oligonucleotide comprises the sequence CTGTCCTAAACCC-GATTCAAGGTTTCAGGGTTAGATGC.
54. The probe of claim 49 wherein said oligonucleotide comprise the sequence AGGCACTGTCCC-TAAACCCGATTCAAGGTT.
55. The probe of claim 49 wherein said oligonucleotide comprises the sequence CCGCTAAAGCGCTT-
- 40 TCCACCACAAAGAACATGCATCCCG.
56. The probe of claim 49 wherein said oligonucleotide comprise the sequence ACACCGCTAAAGCGC-TTTCCACCAAGAACATGCATC.
57. A nucleotide polymer capable of hybridizing to the probe of claims 50 or 51 or 52 or 53 or 54 or 55 or 56 or to the complement thereof.
- 45 58. A nucleic acid hybrid formed between an oligonucleotide comprising a member of the group consisting of oligonucleotides of the sequences  
TAAAGCGCTTCCACCAAGAACATGCATCCCGTG,  
TGCCCTACCCACACCCACCAAGGTGATGT,  
CCATCACCAACCCCTCCTCCGGAGAGGAAAAGG,  
50 CTGTCCTAAACCCGATTCAAGGTTCGAGGTTAGATGC,  
AGGCACTGTCCCTAAACCCGATTCAAGGTT,  
CCGCTAAAGCGCTTCCACCAAGAACATGCATCCCG, and  
ACACCGCTAAAGCGCTTCCACCAAGAACATGCATC;  
and a nucleic acid sequence substantially complementary thereto.
- 55 59. A nucleotide polymer comprising a member of the group consisting of nucleotide polymers of the structures  
TAAAGCGCTTCCACCAAGAACATGCATCCCGTG,  
TGCCCTACCCACACCCACCAAGGTGATGT.

CCATCACCAACCTCCTCCGGAGAGGAAAAGG,  
 CTGTCCCTAAACCGATTCAAGGTTGAGGTTAGATGC,  
 AGGCACTGTCCCTAAACCGATTCAAGGTTTC,  
 CCGCTAAAGCGCTTCCACCACAAGACATGCATCCCG, and  
 5 ACACCGCTAAAGCGCTTCCACCACAAGACATGCATC;

and the complements thereto.

- 60. A nucleotide polymer capable of hybridizing to the rRNA of the species included in the Mycobacterium tuberculosis complex in the region corresponding to bases 185-225 of E. coli 16S rRNA.
- 61. A nucleic acid hybrid formed between a nucleotide polymer of claim 60 and a nucleic acid substantially complementary thereto.
- 62. A nucleotide polymer capable of hybridizing to the RNA of the species included in the Mycobacterium tuberculosis complex in the region corresponding to bases 540-575 of E. coli 23S rRNA.
- 63. A nucleic acid hybrid formed between a nucleotide polymer of claim 62 and a nucleic acid substantially complementary thereto.
- 64. A nucleotide polymer capable of hybridizing to the RNA of the species included in the Mycobacterium tuberculosis complex in the region corresponding to bases 1155-1190 of E. coli 23S rRNA.
- 65. A nucleic acid hybrid formed between a nucleotide polymer of claim 64 and a nucleic acid substantially complementary thereto.
- 66. A nucleotide polymer capable of hybridizing to the RNA of the species included in the Mycobacterium tuberculosis complex in the region corresponding to bases 2195-2235 of E. coli 23S rRNA.
- 67. A nucleic acid hybrid formed between a nucleotide polymer of claim 66 and a nucleic acid substantially complementary thereto.
- 68. The probe of claim 31 wherein said non-viral organisms are the genus Mycobacterium.
- 69. The probe of claim 68 wherein said oligonucleotide comprises the sequence CCA TGC ACC ACC
- 70. The probe of claim 68 wherein said oligonucleotide comprises the sequence GGC TTG CCC CAG TAT TAC CAC TGA CTG GTA CGG.
- 71. The probe of claim 68 wherein said oligonucleotide comprises the sequence CAC CGA ATT CGC CTC AAC CGG CTA TGC ACC TC.
- 72. The probe of claim 68 wherein said oligonucleotide comprises the sequence GGG GTA CGG CCC GTG TGT GTG CTC GCT AGA GGC.
- 73. A nucleotide polymer capable of hybridizing to the probe of claims 69 or 70 or 71 or 72 or to the complement thereof.
- 74. A nucleic acid hybrid formed between an oligonucleotide comprising a member of the group consisting of oligonucleotides of the sequence
- 75. A nucleotide polymer comprising a member of the group consisting of nucleotide polymers of the structures
- 76. A nucleotide polymer capable of hybridizing to RNA of the genus Mycobacterium in the region corresponding to bases 1025-1080 of E. coli 16S rRNA.
- 77. A nucleic acid hybrid formed between a nucleotide polymer of claim 76 and a nucleic acid sequence substantially complementary thereto.
- 78. A nucleotide polymer capable of hybridizing to RNA of the genus Mycobacterium in the region corresponding to bases 1440-1475 of E. coli 23S rRNA.
- 79. A nucleic acid hybrid formed between a nucleotide polymer of claim 78 and a nucleic acid sequence substantially complementary thereto.

80. A nucleotide polymer capable of hybridizing to RNA of the genus Mycobacterium in the region corresponding to bases 1515-1555 of E. coli 23S rRNA.
81. A nucleic acid hybrid formed between a nucleotide polymer of claim 80 and a nucleic acid sequence substantially complementary thereto.
- 5 82. A nucleotide polymer capable of hybridizing to RNA of the genus Mycobacterium in the region corresponding to bases 1570-1610 of E. coli 23S rRNA.
83. A nucleic acid hybrid formed between a nucleotide polymer of claim 82 and a nucleic acid sequence substantially complementary thereto.
84. The probe of claim 31 wherein said non-viral organism is Mycoplasma pneumoniae.
- 10 85. The probe of claim 84 wherein said oligonucleotide comprises the sequence  
GCTTGGTGCTTCCTATTCTCACTGAAACAGCTACATTCGGC.
86. The probe of claim 84 wherein said oligonucleotide comprises the sequence  
AATAACGAACCCTTGCAGGTCTTCAACTTTGAT.
87. The probe of claim 84 wherein said oligonucleotide comprises the sequence CAGTCAAACTCTAG-
- 15 CCATTACCTGCTAAAGTCATT.
88. The probe of claim 84 wherein said oligonucleotide comprise the sequence  
TACCGAGGGGATCGCCCCGACAGCTAGTAT.
89. The probe of claim 84 wherein said oligonucleotide comprises the sequence  
CTTTACAGATTTGCTCACTTTACAAGCTGGCGAC.
- 20 90. A nucleotide polymer capable of hybridizing to the probes of claims 85 to 86 or 87 or 88 or 89 or to the complement thereof.
91. A nucleic acid hybrid formed between an oligonucleotide comprising a member of the group consisting of oligonucleotides of the sequence  
GCTTGGTGCTTCCTATTCTCACTGAAACAGCTACATTCGGC,
- 25 AATAACGAACCCTTGCAGGTCTTCAACTTTGAT,  
CAGTCAAACTCTAGCCATTACCTGCTAAAGTCATT,  
TACCGAGGGGATCGCCCCGACAGCTAGTAT, and  
CTTTACAGATTTGCTCACTTTACAAGCTGGCGAC;
- and a nucleic acid sequence substantially complementary thereto.
- 30 92. A nucleotide polymer comprising a member of the group consisting of nucleotide polymers of the structures  
GCTTGGTGCTTCCTATTCTCACTGAAACAGCTACATTCGGC,  
AATAACGAACCCTTGCAGGTCTTCAACTTTGAT,  
CAGTCAAACTCTAGCCATTACCTGCTAAAGTCATT,  
35 TACCGAGGGGATCGCCCCGACAGCTAGTAT, and  
CTTTACAGATTTGCTCACTTTACAAGCTGGCGAC;
- and the complements thereto.
93. A nucleotide polymer capable of hybridizing to the RNA of the species Mycoplasma pneumoniae in the region corresponding to bases 190-230 of E. coli 16S rRNA.
- 40 94. A nucleic acid hybrid formed between a nucleotide polymer of claim 93 and a nucleic acid sequence substantially complementary thereto.
- A nucleotide polymer capable of hybridizing to the RNA of the species Mycoplasma pneumoniae in the region corresponding to bases 450-490 of E. coli 16S rRNA.
95. A nucleic acid hybrid formed between a nucleotide polymer of claim 95 and a nucleic acid sequence substantially complementary thereto.
- 45 96. A nucleic acid hybrid formed between a nucleotide polymer of claim 97 and a nucleic acid sequence substantially complementary thereto.
97. A nucleotide polymer capable of hybridizing to the RNA of the species Mycoplasma pneumoniae in the region corresponding to bases 820-860 of E. coli 16S rRNA.
98. A nucleic acid hybrid formed between a nucleotide polymer of claim 97 and a nucleic acid sequence substantially complementary thereto.
- 50 99. A nucleotide polymer capable of hybridizing to the RNA of the species Mycoplasma pneumoniae in the region corresponding to bases 1255-1290 of E. coli 16S rRNA.
100. A nucleic acid hybrid formed between a nucleotide polymer of claim 99 and a nucleic acid sequence substantially complementary thereto.
101. A nucleotide polymer capable of hybridizing to the RNA of the species Mycoplasma pneumoniae
- 55 In the region corresponding to bases 65-120 of E. coli 5S rRNA.
102. A nucleic acid hybrid formed between a nucleotide polymer of claim 101 and a nucleic acid sequence substantially complementary thereto.
103. The probe of claim 31 wherein said non-viral organisms are the genus Legionella.

104. The probe of claim 103 wherein said oligonucleotide comprises the sequence TACCCCTCTCC-CATACTCGAGTCAACCAGTATTATCTGACC.
105. The probe of claim 103 wherein said oligonucleotide comprises the sequence GGATTTCACGTG-TCCCCGCCTACTTGGTTCGGGTGCGTAGTT.
- 5 106. The probe of claim 103 wherein said oligonucleotide comprises the sequence CATCTCTG-CAAAATTCACTGTATGTCAAGGGTAGGTAAGG.
107. The probe of claim 103 wherein said oligonucleotide comprises the sequence GCGGTACGGTTC-TCTATAAGTTATGGCTAGC.
108. The probe of claim 103 wherein said oligonucleotide comprises the sequence GTACCGAGGG-10 TACCTTTGTGCT.
109. The probe of claim 103 wherein said oligonucleotide comprises the sequence CACTCTGGTAC-GATGTCCGAC.
110. A nucleotide polymer capable of hybridizing to the probes of claims 104 or 105 or 106 or 107 or 108 or 109 or to the complement thereof.
- 15 111. A nucleic acid hybrid formed between an oligonucleotide comprising a member of the group consisting of oligonucleotides of the sequences  
 TACCCCTCTCCCATACTCGAGTCAACCAGTATTATCTGACC,  
 GGATTTCACGTGTCGGCCTACTTGGTTCGGGTGCGTAGTT,  
 CATCTCTGCAAAATTCACTGTATGTCAAGGGTAGGTAAGG,  
 20 GCTGTACGGTTCTCTATAATGTATGGCTAGC,  
 GTACCGAGGGTACCTTGCT, and  
 CACTCTGGTACGATGTCCGAC;  
 and a nucleic acid sequence substantially complementary thereto.
112. A nucleotide polymer comprising a member of the group consisting of nucleotide polymers of the 25 structures  
 TACCCCTCTCCCATACTCGAGTCAACCAGTATTATCTGACC,  
 GGATTTCACGTGTCGGCCTACTTGGTTCGGGTGCGTAGTT,  
 CATCTCTGCAAAATTCACTGTATGTCAAGGGTAGGTAAGG,  
 GCGGTACGGTTCTCTATAAGTTATGGCTAGC,  
 30 GTACCGAGGGTACCTTGCT, and  
 CACTCTGGTACGATGTCCGAC;  
 and the complements thereto.
113. A nucleotide polymer capable of hybridizing to the RNA of the genus Legionella in the region corresponding to bases 630-675 of E. coli 16S rRNA.
- 35 114. A nucleic acid hybrid formed between a nucleotide polymer of claim 113 and a nucleic acid sequence substantially complementary thereto.
115. A nucleotide polymer capable of hybridizing to the RNA of the genus Legionella in the region corresponding to bases 975-1020 of E. coli 16S rRNA.
- 40 116. A nucleic acid hybrid formed between a nucleotide polymer of claim 115 and a nucleic acid sequence substantially complementary thereto.
117. A nucleotide polymer capable of hybridizing to the RNA of the genus Legionella in the region corresponding to bases 350-395 of E. coli 23S rRNA.
118. A nucleic acid hybrid formed between a nucleotide polymer of claim 117 and a nucleic acid sequence substantially complementary thereto.
- 45 119. A nucleotide polymer capable of hybridizing to the RNA of the genus Legionella in the region corresponding to bases 1585-1620 of E. coli 23S rRNA.
120. A nucleic acid hybrid formed between a nucleotide polymer of claim 119 and a nucleic acid sequence substantially complementary thereto.
- 50 121. A nucleotide polymer capable of hybridizing to the RNA of the genus Legionella in the region corresponding to bases 2280-2330 of E. coli 23S rRNA.
122. A nucleic acid hybrid formed between a nucleotide polymer of claim 121 and a nucleic acid sequence substantially complementary thereto.
123. The probe of claim 31 wherein said non-viral organism is Chlamydia trachomatis.
- 55 124. The probe of claim 123 wherein said oligonucleotide comprises the sequence  
 CCGACTCGGGTTGAGCCCATTTGACAA.  
 125. The probe of claim 123 wherein said oligonucleotide comprises the sequence  
 TTACGTCCGACACGGATGGGGTTGAGACCATC.

104. The probe of claim 103 wherein said oligonucleotide comprises the sequence TACCCTCTCC-CATACTCGAGTCACCAAGTATTATCTGACC.
105. The probe of claim 103 wherein said oligonucleotide comprises the sequence GGATTTCACGTG-TCCCCGCCTACTTGTTCGGGTGCGTAGTT.
- 5 106. The probe of claim 103 wherein said oligonucleotide comprises the sequence CATCTCTG-CAAAATTCACTGTATGTCAAGGGTAGGTAAGG.
107. The probe of claim 103 wherein said oligonucleotide comprises the sequence GCGGTACGGTTC-TCTATAAGTTATGGCTAGC.
108. The probe of claim 103 wherein said oligonucleotide comprises the sequence GTACCGAGGG-10 TACCTTTGTGCT.
109. The probe of claim 103 wherein said oligonucleotide comprises the sequence CACTCTGGTAC-GATGTCCGAC.
110. A nucleotide polymer capable of hybridizing to the probes of claims 104 or 105 or 106 or 107 or 108 or 109 or to the complement thereof.
- 15 111. A nucleic acid hybrid formed between an oligonucleotide comprising a member of the group consisting of oligonucleotides of the sequences  
 TACCCTCTCCCATACTCGAGTCACCAAGTATTATCTGACC,  
 GGATTTACGTGTCCCCGCCTACTTGTTCGGGTGCGTAGTT,  
 CATCTCTGCAAAATTCACTGTATGTCAAGGGTAGGTAAGG,  
 20 GCTGTACGGTTCTCTATAATGTATGGCTAGC,  
 GTACCGAGGGTACCTTTGTGCT, and  
 CACTCTGGTACGATGTCCGAC;  
 and a nucleic acid sequence substantially complementary thereto.
112. A nucleotide polymer comprising a member of the group consisting of nucleotide polymers of the 25 structures  
 TACCCTCTCCCATACTCGAGTCACCAAGTATTATCTGACC,  
 GGATTTACGTGTCCCCGCCTACTTGTTCGGGTGCGTAGTT,  
 CATCTCTGCAAAATTCACTGTATGTCAAGGGTAGGTAAGG,  
 GCGGTACGGTTCTCTATAAGTTATGGCTAGC,  
 30 GTACCGAGGGTACCTTTGTGCT, and  
 CACTCTGGTACGATGTCCGAC;  
 and the complements thereto.
113. A nucleotide polymer capable of hybridizing to the RNA of the genus Legionella in the region corresponding to bases 630-675 of E. coli 16S rRNA.
- 35 114. A nucleic acid hybrid formed between a nucleotide polymer of claim 113 and a nucleic acid sequence substantially complementary thereto.
115. A nucleotide polymer capable of hybridizing to the RNA of the genus Legionella in the region corresponding to bases 975-1020 of E. coli 16S rRNA.
- 40 116. A nucleic acid hybrid formed between a nucleotide polymer of claim 115 and a nucleic acid sequence substantially complementary thereto.
117. A nucleotide polymer capable of hybridizing to the RNA of the genus Legionella in the region corresponding to bases 350-395 of E. coli 23S rRNA.
118. A nucleic acid hybrid formed between a nucleotide polymer of claim 117 and a nucleic acid sequence substantially complementary thereto.
- 45 119. A nucleotide polymer capable of hybridizing to the RNA of the genus Legionella in the region corresponding to bases 1585-1620 of E. coli 23S rRNA.
120. A nucleic acid hybrid formed between a nucleotide polymer of claim 119 and a nucleic acid sequence substantially complementary thereto.
- 50 121. A nucleotide polymer capable of hybridizing to the RNA of the genus Legionella in the region corresponding to bases 2280-2330 of E. coli 23S rRNA.
122. A nucleic acid hybrid formed between a nucleotide polymer of claim 121 and a nucleic acid sequence substantially complementary thereto.
123. The probe of claim 31 wherein said non-viral organism is Chlamydia trachomatis.
- 55 124. The probe of claim 123 wherein said oligonucleotide comprises the sequence  
 CCGACTCGGGTTGAGCCCATTTGACAA.
125. The probe of claim 123 wherein said oligonucleotide comprises the sequence  
 TTACGTCCGACACGGATGGGGTTGAGACCATC.

126. The probe of claim 123 wherein said oligonucleotide comprises the sequence CGGCCACTAAACAATCGTCGAAACAATTGCTCCGTTCGA.
127. The probe of claim 123 wherein said oligonucleotide comprises the sequence CGTTACTCGGATGCCAAATATGCCACATTG.
- 5 128. The probe of claim 123 wherein said oligonucleotide comprises the sequence CATCCATCTTCCAGATGTGTTCAACTAGGAGTCCTGATCC.
129. The probe of claim 123 wherein said oligonucleotide comprises the sequence GAGGTCGGTCTTCTCCTTCGTCTACG.
130. The probe of claim 123 wherein said oligonucleotide comprises the sequence 10 CCGTTCTCATCGCTCTACGGACTCTTCCAATCG.
131. The probe of claim 123 wherein said oligonucleotide comprises the sequence CGAAGATTCCCCTTGATCGCGACCTGATCT.
132. The probe of claim 123 wherein said oligonucleotide comprises the sequence CCGGGGCTCCTATCGTTCCATAGTCACCCCTAAAAG.
- 15 133. The probe of claim 123 wherein said oligonucleotide comprises the sequence TACCGCGTGTCTTATCGACACACCCGCG.
134. A nucleotide polymer capable of hybridizing to the probe of claims 124 or 125 or 126 or 127 or 128 or 129 or 130 or 131 or 132 or 133 or to the complement thereof.
135. A nucleic acid hybrid formed between an oligonucleotide comprising a member of the group 20 consisting of oligonucleotides of the sequence
- CCGACTCGGGGTTGAGCCATCTTGACAA,  
 TTACGTCCGACACGGATGGGTTGAGACCATC,  
 CCGCCACTAAACAATCGTCGAAACAATTGCTCCGTTCGA,  
 CGTTACTCGGATGCCAAATATGCCACATTG,  
 25 CATCCATCTTCCAGATGTGTTCAACTAGGAGTCCTGATCC,  
 GAGGTCGGTCTTCTCCTCCCTCGTCTACG,  
 CCGTTCTCATCGCTCTACGGACTCTTCCAATCG,  
 CGAAGATTCCCCTTGATCGCGACCTGATCT,  
 CCGGGGCTCCTATCGTTCCATAGTCACCCCTAAAAG, and TACCGCGTGTCTTATC-  
 30 GACACACCCCGCG;  
 and a nucleic acid sequence substantially complementary thereto.
136. A nucleotide polymer comprising a member of the group consisting of nucleotide polymers of the structures
- 35 CCGACTCGGGGTTGAGCCATCTTGACAA,  
 TTACGTCCGACACGGATGGGTTGAGACCATC,  
 CCGCCACTAAACAATCGTCGAAACAATTGCTCCGTTCGA,  
 CGTTACTCGGATGCCAAATATGCCACATTG,  
 CATCCATCTTCCAGATGTGTTCAACTAGGAGTCCTGATCC,  
 40 GAGGTCGGTCTTCTCCTCCCTCGTCTACG,  
 CCGTTCTCATCGCTCTACGGACTCTTCCAATCG,  
 CGAAGATTCCCCTTGATCGCGACCTGATCT,  
 CCGGGGCTCCTATCGTTCCATAGTCACCCCTAAAAG, and  
 TACCGCGTGTCTTATCGACACACCCCGCG;
- and the complements thereto.
- 45 137. A nucleotide polymer capable of hybridizing to the RNA of the species Chlamydia trachomatis in the region corresponding to bases 80-105 of E. coli 16S rRNA.
138. A nucleic acid hybrid formed between a nucleotide polymer of claim 137 and a nucleic acid substantially complementary thereto.
- 50 139. A nucleotide polymer capable of hybridizing to the RNA of the species Chlamydia trachomatis in the region corresponding to bases 175-210 of E. coli 16S rRNA.
140. A nucleic acid hybrid formed between a nucleotide polymer of claim 138 and a nucleic acid substantially complementary thereto.
- 55 141. A nucleotide polymer capable of hybridizing to the RNA of the species Chlamydia trachomatis in the region corresponding to bases 600-635 of E. coli 16S rRNA.
142. A nucleic acid hybrid formed between a nucleotide polymer of claim 141 and a nucleic acid substantially complementary thereto.
143. A nucleotide polymer capable of hybridizing to the RNA of the species Chlamydia trachomatis in the region corresponding to bases 830-870 of E. coli 16S rRNA.

168. A nucleic acid hybrid formed between a nucleotide polymer of claim 167 and a nucleic acid sequence complementary thereto.
169. A nucleotide polymer capable of hybridizing to the RNA of the genus Campylobacter in the region corresponding to bases 705-735 of E. coli 16S rRNA.
- 5 170. A nucleic acid hybrid formed between a nucleotide polymer of claim 169 and a nucleic acid substantially complementary thereto.
171. A nucleotide polymer capable of hybridizing to the RNA of the genus Campylobacter in the region corresponding to bases 980-1010 of E. coli 16S rRNA.
- 10 172. A nucleic acid hybrid formed between a nucleotide polymer of claim 171 and a nucleic acid sequence substantially complementary thereto.
173. The probe of claim 31 wherein said non-viral organisms are the sub-generic group of Streptococci known as enterococci.
174. The probe of claim 173 wherein said oligonucleotide comprises the sequence TGC AGC ACT GAA GGG CGG AAA CCC TCC AAC ACT TA.
- 15 175. A nucleotide polymer capable of hybridizing to the probe of claim 174 or to the complement thereof.
176. A nucleic acid hybrid formed between an oligonucleotide comprising the sequence  
TGC AGC ACT GAA GGG CGG AAA CCC TCC AAC ACT TA and a nucleic acid sequence substantially complementary.
- 20 177. A nucleotide polymer of the structure  
TGC AGC ACT GAA GGG CGG AAA CCC TCC AAC ACT TA and the complement thereto.
178. A nucleotide polymer capable of hybridizing to the RNA of the sub-generic group Streptococci known as enterococci in the region corresponding to bases 825-860 of E. coli 16S rRNA.
- 25 179. A nucleic acid hybrid formed between a nucleotide polymer of claim 178 and a nucleic acid sequence substantially complementary thereto.
180. The probe of claim 31 wherein said non-viral organisms are the subgeneric grouping known as Group I Pseudomonas.
181. The probe of claim 180 wherein said oligonucleotide comprises the sequence  
CAG ACA AAG TTT CTC GTG CTC CGT CCT ACT CGA TT.
- 30 182. A nucleotide polymer capable of hybridizing to the probe of claim 181 or to the complement thereof.
183. A nucleic acid hybrid formed between an oligonucleotide comprising the sequence  
CAG ACA AAG TTT CTC GTG CTC CGT CCT ACT CGA TT  
and a nucleic acid substantially complementary thereto.
- 35 184. A nucleotide polymer of the structure  
CAG ACA AAG TTT CTC GTG CTC CGT CCT ACT CGA TT  
and the complement thereto.
185. A nucleotide polymer capable of hybridizing to the RNA of the sub-generic grouping known as group I Pseudomonas in the region corresponding to the bases 365-405 of E. coli 23S rRNA.
- 40 186. A nucleic acid hybrid formed between a nucleotide polymer of claim 185 and a nucleic acid sequence substantially complementary thereto.
187. The probe of claim 31 wherein said non-viral organism is Enterobacter cloacae.
188. The probe of claim 187 wherein said oligonucleotide comprises the sequence  
GTG TGT TTT CGT GTA CGG GAC TTT CAC CC.
- 45 189. A nucleotide polymer capable of hybridizing to the probe of claim 188 or to the complement thereof.
190. A nucleic acid hybrid formed between an oligonucleotide comprising a member of the group consisting of oligonucleotides of the sequence  
GTG TGT TTT CGT GTA CGG GAC TTT CAC CC
- 50 50 and a nucleic acid sequence substantially complementary thereto.
191. A nucleotide polymer of the structure  
GTG TGT TTT CGT GTA CGG GAC TTT CAC CC  
and the complement thereto.
192. A nucleotide polymer capable of hybridizing to the RNA of the species Enterobacter cloacae in the region corresponding to bases 305-340 of E. coli 23S rRNA.
- 55 193. A nucleic acid hybrid formed between a nucleotide polymer of claim 192 and a nucleic acid sequence substantially complementary thereto.
194. The probe of claim 31 wherein said non-viral organism is Proteus mirabilis.

242. The probe of claim 241 wherein said oligonucleotide comprises the sequence  
 CCC GAC CGT CCC TAT TAA TCA TTA CGA TGG.
243. The probe of claim 241 wherein said oligonucleotide comprise the sequence CCCGACCGTCCC-TATTAATCATTACGATGGTCCTAGAAAC.
- 5 244. The probe of claim 241 wherein said oligonucleotide comprises the sequence CCCGACCGTCCC-TATTAATCATTACGATGG.
245. The probe of claim 241 wherein said oligonucleotide comprises the sequence  
 CGA CTT GGC ATG AAA ACT ATT CCT TCC TGT GG.
- 10 246. The probe of claim 241 wherein said oligonucleotide comprises the sequence  
 GCT CTT CAT TCA ATT GTC CAC GTT CAA TTA AGC AAC AAG G.
247. The probe of claim 241 wherein said oligonucleotide comprises the sequence  
 GCT CTG CAT TCA AAG GTC CGC GTT CAA TAA AGA AAC AGG G.
248. A nucleotide polymer capable of hybridizing to the probe of claims 242 or 243 or 244 or 245 or  
 246 or 247 or to the complement thereof.
- 15 249. A nucleic acid hybrid formed between an oligonucleotide comprising a member of the group  
 consisting of oligonucleotides of the sequence  
 CCC GAC CGT CCC TAT TAA TCA TTA CGA TGG,  
 CCCGACCGTCCCCTATTAATCATTACGATGGTCCTAGAAAC  
 CCCGACCGTCCCCTATTAATCATTACGATGG  
 20 CGA CTT GGC ATG AAA ACT ATT CCT TCC TAT GG,  
 GCT CTT CAT TCA ATT GTC CAC GTT CAA TTA AGC AAC AGG G, and  
 GCT CTG CAT TCA AAC GTC CGC GTT CAA TAA AGA AAC AGG G;  
 and a nucleic acid sequence substantially complementary thereto.
- 25 250. A nucleotide polymer comprising a member of the group consisting of nucleotide polymers of the  
 structures  
 CCC GAC CGT CCC TAT TAA TCA TTA CGA TGG,  
 CCCGACCGTCCCCTATTAATCATTACGATGGTCCTAGAAAC  
 CCCGACCGTCCCCTATTAATCATTACGATGG  
 CGA CTT GGC ATG AAA ACT ATT CCT TCC TAT GG,  
 30 GCT CTT CAT TCA ATT GTC CAC GTT CAA TTA AGC AAC AGG G, and  
 GCT CTG CAT TCA AAC GTC CGC GTT CAA TAA AGA AAC AGG G;  
 and the complements thereto.
251. A nucleotide polymer capable of hybridizing to the RNA of the phylogenetic group Fungi in the  
 region corresponding to position 845-880 of Saccharomyces cerevisiae 18S rRNA.
- 35 252. A nucleic acid hybrid formed between a nucleotide polymer of claim 251 and a nucleic acid  
 sequence substantially complementary thereto.
253. A nucleotide polymer capable of hybridizing to the RNA of the phylogenetic group Fungi in the  
 region corresponding to position 1980-2000 of Saccharomyces cerevisiae 28S rRNA.
- 40 254. A nucleic acid hybrid formed between a nucleotide polymer of claim 253 and a nucleic acid  
 substantially complementary thereto.
255. A nucleotide polymer capable of hybridizing to the RNA of the phylogenetic group Fungi in the  
 region corresponding to position 1225-1270 of Saccharomyces cerevisiae 28S rRNA.
256. A nucleic acid hybrid formed between a nucleotide polymer of claim 255 and a nucleic acid  
 substantially complementary thereto.
- 45 257. The probe of claim 31 wherein said non-viral organism is Neisseria gonorrhoeae.
258. The probe of claim 257 wherein said oligonucleotide comprises the sequence  
 CCG CCG CTA CCC GGT AC.
259. The probe of claim 257 wherein said oligonucleotide comprises the sequence  
 TCA TCG GCC GCC GAT ATT GGC.
- 50 260. The probe of claim 257 wherein said oligonucleotide comprises the sequence  
 GAG CAT TCC GCA CAT GTC AAA ACC AGG TA.
261. The probe of claim 257 wherein said oligonucleotide comprises the sequence  
 GAG GAT TCC GCA CAT GTC AAA ACC AGG.
- 55 262. The probe of claim 257 wherein said oligonucleotide comprises the sequence  
 GAG GAT TCC GCA CAT GTC AAA ACC AGG TAA.
263. The probe of claim 257 wherein said oligonucleotide comprises the sequence  
 CCC GCT ACC CGG TAC GTTC.

284. The probe of claim 257 wherein said oligonucleotide comprises the sequence  
 CCG CTA CCC GGTAC GTTC.
285. A nucleotide polymer capable of hybridizing to the probe of claims 258 or 259 or 260 or 261 or 262 or 263 or 264 or to the complement thereof.
- 5 266. A nucleic acid hybrid formed between an oligonucleotide comprising a member of the group consisting of oligonucleotides of the sequences  
 CCGCCGCTACCCGGTAC,  
 TCATCGGCCGCCGATATTGGC,  
 GAGCATTCCGCACATGTCAAAACCAGGTA,  
 10 GAGGATTCCGCACATGTCAAAACCAGG,  
 GAGGATTCCGCACATGTCAAAACCAGGTA,  
 CCCGCTACCCGGTACGTTC, and  
 CCGCTACCCGGTACGTTC;  
 and a nucleic acid sequence substantially complementary thereto.
- 15 267. A nucleotide polymer comprising a member of the group consisting of nucleotide polymers of the structures  
 CCGCCGCTACCCGGTAC,  
 TCATCGGCCGCCGATATTGGC,  
 GAGCATTCCGCACATGTCAAAACCAGGTA,  
 20 GAGGATTCCGCACATGTCAAAACCAGG,  
 GAGGATTCCGCACATGTCAAAACCAGGTA,  
 CCCGCTACCCGGTACGTTC, and  
 CCGCTACCCGGTACGTTC;  
 and the complements thereto.
- 25 268. A nucleotide polymer capable of hybridizing to the RNA of the species Neisseria gonorrhoeae in the region corresponding to bases 125-150 of E. coli 16s rRNA.
269. A nucleic acid hybrid formed between a nucleotide polymer of claim 268 and a nucleic acid sequence substantially complementary thereto.
270. A nucleotide polymer capable of hybridizing to the RNA of the species Neisseria gonorrhoeae in the region corresponding to bases 455-485 of E. coli 16s rRNA.
- 30 271. A nucleic acid hybrid formed between a nucleotide polymer of claim 270 and a nucleic acid sequence substantially complementary thereto.
272. A nucleotide polymer capable of hybridizing to the RNA of the species Neisseria gonorrhoeae in the region corresponding to bases 980-1015 of E. coli 16s rRNA.
- 35 273. A nucleic acid hybrid formed between a nucleotide polymer of claim 272 and a nucleic acid sequence substantially complementary thereto.
274. The probe of claim 31 wherein said oligonucleotide is perfectly complementary to said region of rRNA.
275. The probe of claim 31 wherein said oligonucleotide is about 20 nucleotides to about 50 nucleotides 40 in length.
276. The probe of claim 31 wherein said oligonucleotide is at least about 95% complementary to a region of rRNA.
277. A hybridization assay comprising (1) reacting together any rRNA from a sample to be assayed for a non-viral organism or organisms and an oligonucleotide probe of at least about 10 nucleotides in length 45 which is at least about 75% complementary to a variable region of rRNA selected to be unique to said non-viral organism or organisms, (2) under conditions such that hybridization between the oligonucleotide probe and any sufficiently complementary sample rRNA can occur, and (3) observing and/or measuring said hybridization.
278. The assay of claim 277 wherein said hybridization between the oligonucleotide probe and any 50 target sample rRNA is from at least about 10% to about 100%.
279. The assay of claim 277 wherein said oligonucleotide probe is cDNA.
280. The assay of claim 277 wherein said conditions include a temperature from about 25°C below T<sub>m</sub> to about 1°C below T<sub>m</sub>.
281. The assay of claim 277 which further comprises the parallel assay of a positive homologous 55 control, or a positive heterologous control, or both.
282. The assay of claim 277 which further comprises the parallel assay of a negative control.
283. The assay of claim 277 wherein said conditions include agents for increased rates of hybridization.

323. A nucleic acid hybrid formed between a nucleotide polymer of claim 322 and a nucleic acid sequence substantially complementary thereto.

324. A probe consisting of a nucleotide polymer which is capable of hybridizing to 23S like rRNA of a nonviral organism or group of organisms in the region corresponding to bases 1440-1600 of E.Coli 23S rRNA.

325. A nucleic acid hybrid formed between a nucleotide polymer of claim 324 and a nucleic acid sequence substantially complementary thereto.

326. A probe consisting of a nucleotide polymer which is capable of hybridizing to 23S like rRNA of a nonviral organism or group of organisms in the region corresponding to bases 1710-1750 of E.Coli 23S rRNA.

327. A nucleic acid hybrid formed between a nucleotide polymer of claim 326 and a nucleic acid sequence substantially complementary thereto.

328. A probe consisting of a nucleotide polymer which is capable of hybridizing to 23S like rRNA of a nonviral organism or group of organisms in the region corresponding to bases 2180-2330 of E.Coli 23S rRNA.

329. A nucleic acid hybrid formed between a nucleotide polymer of claim 328 and a nucleic acid sequence substantially complementary thereto.

20

25

30

35

40

45

50

55

FIG. 1

FIG. 2

0 272 009

Fachverzeichnisse

100 12

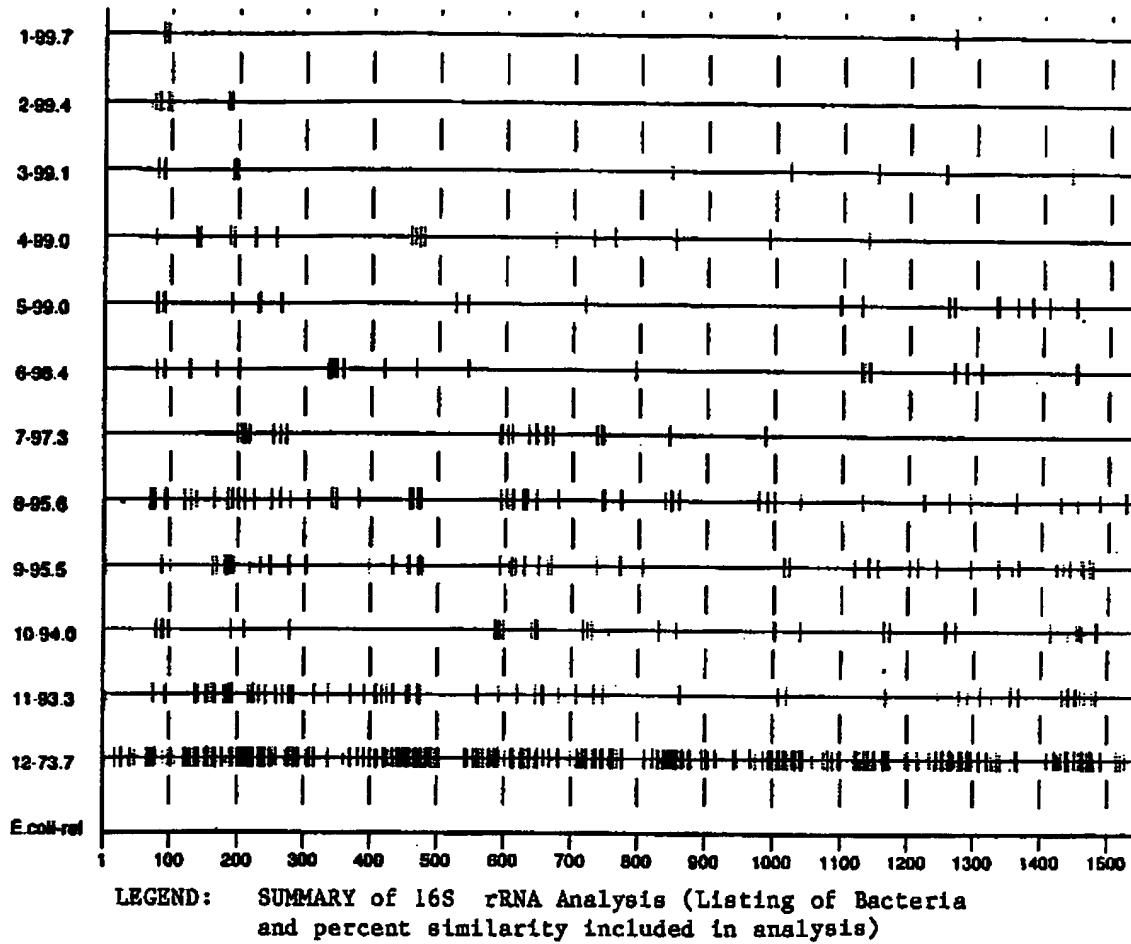
FIG. 3

S. cerevisiae	sc	UACUCGTCGAAUCCUGCGGGGUCAUAGCTCUCAAAGAUUACGCCAUGCUCAGCUAGCAUUAACAGGAAACGCAAGCUCAGGAA	100
S. cerevisiae	sc	UUAAACGCUAAUCGUAUUCGUAUAGCUAUCGCUAUCGCUAUCGCUAUCGCUAUCGCUAUCGCUAUCGCUAUCGCUAUCGCUA	200
S. cerevisiae	sc	GAUCCAUUUAUAGAUAAAACUACAUUACGUUACGUUACGUUACGUUACGUUACGUUACGUUACGUUACGUUACGUUACGUUACGUU	300
S. cerevisiae	sc	AUCGUUACGUUACGUUACGUUACGUUACGUUACGUUACGUUACGUUACGUUACGUUACGUUACGUUACGUUACGUUACGUUACGUU	400
S. cerevisiae	sc	ACGCCAACACACGCCAACACGCCAACACGCCAACACGCCAACACGCCAACACGCCAACACGCCAACACGCCAACACGCCAACACGCC	500
S. cerevisiae	sc	UUCGUAAUUCGUAAUUCGUAAUUCGUAAUUCGUAAUUCGUAAUUCGUAAUUCGUAAUUCGUAAUUCGUAAUUCGUAAUUCGUAAU	600
S. cerevisiae	sc	AUUACGUAAUUCGUAAUUCGUAAUUCGUAAUUCGUAAUUCGUAAUUCGUAAUUCGUAAUUCGUAAUUCGUAAUUCGUAAUUCGUAAU	700
S. cerevisiae	sc	UCCGUAAUUCGUAAUUCGUAAUUCGUAAUUCGUAAUUCGUAAUUCGUAAUUCGUAAUUCGUAAUUCGUAAUUCGUAAUUCGUAAU	800
S. cerevisiae	sc	GUAAU	900
S. cerevisiae	sc	GUAAU	1000
S. cerevisiae	sc	GUAAU	1100
S. cerevisiae	sc	GUAAU	1200
S. cerevisiae	sc	GUAAU	1300
S. cerevisiae	sc	AAUUGGUUCAUAAUUCGUAAUUCGUAAUUCGUAAUUCGUAAUUCGUAAUUCGUAAUUCGUAAUUCGUAAUUCGUAAUUCGUAAUUCGU	1400
S. cerevisiae	sc	AGGCCAUUCAAUUACGUUACGUUACGUUACGUUACGUUACGUUACGUUACGUUACGUUACGUUACGUUACGUUACGUUACGUUACGUU	1500
S. cerevisiae	sc	ACGCCAUUCAAUUACGUUACGUUACGUUACGUUACGUUACGUUACGUUACGUUACGUUACGUUACGUUACGUUACGUUACGUUACGUU	1600
S. cerevisiae	sc	CAGGUUCCGUAAUUCGUAAUUCGUAAUUCGUAAUUCGUAAUUCGUAAUUCGUAAUUCGUAAUUCGUAAUUCGUAAUUCGUAAUUCGUAAU	1700
S. cerevisiae	sc	GCACGUUACGUAAUUCGUAAUUCGUAAUUCGUAAUUCGUAAUUCGUAAUUCGUAAUUCGUAAUUCGUAAUUCGUAAUUCGUAAUUCGUAAU	1800
S. cerevisiae	sc	UA	1812

FIG. 4

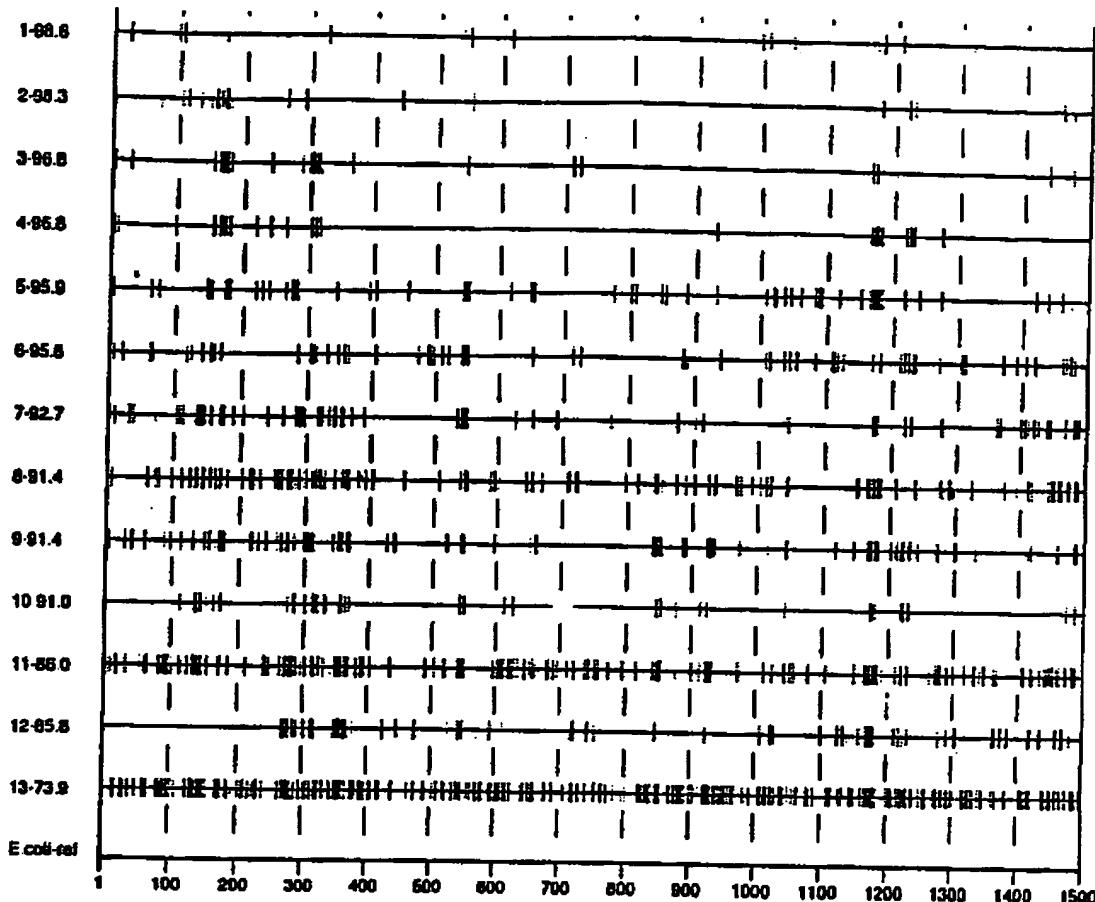
FIG. 5

FIG. 6  
Summary of 16S rRNA Analysis



1. 99.7% *Clostridium botulinum*-*Clostridium subterminale*;
2. 99.4% *Streptococcus cremoris*-*Streptococcus lactis*;
3. 99.1% *Lactobacillus lactis*-*Lactobacillus delbrueckii*;
4. 99.0% *Neisseria gonorrhoeae*-*Neisseria meningitidis*;
5. 99.0% *Mycobacterium intracellulare*-*Mycobacterium avium*;
6. 98.4% *Mycobacterium avium*-*Mycobacterium tuberculosis*;
7. 97.3% *Pseudomonas alcaligenes*-*Pseudomonas stutzeri*;
8. 95.6% *Chlamydia psittaci*-*Chlamydia trachomatis*;
9. 95.5% *Spiroplasma citri*-*Spiroplasma mirum*;
10. 94.0% *Clostridium lituseburense*-*Clostridium sordellii*;
11. 93.3% *Listeria monocytogenes*-*Bacillus thermosphacta*;
12. 73.7% *Escherichia coli*-*Bacteroides fragilis*.

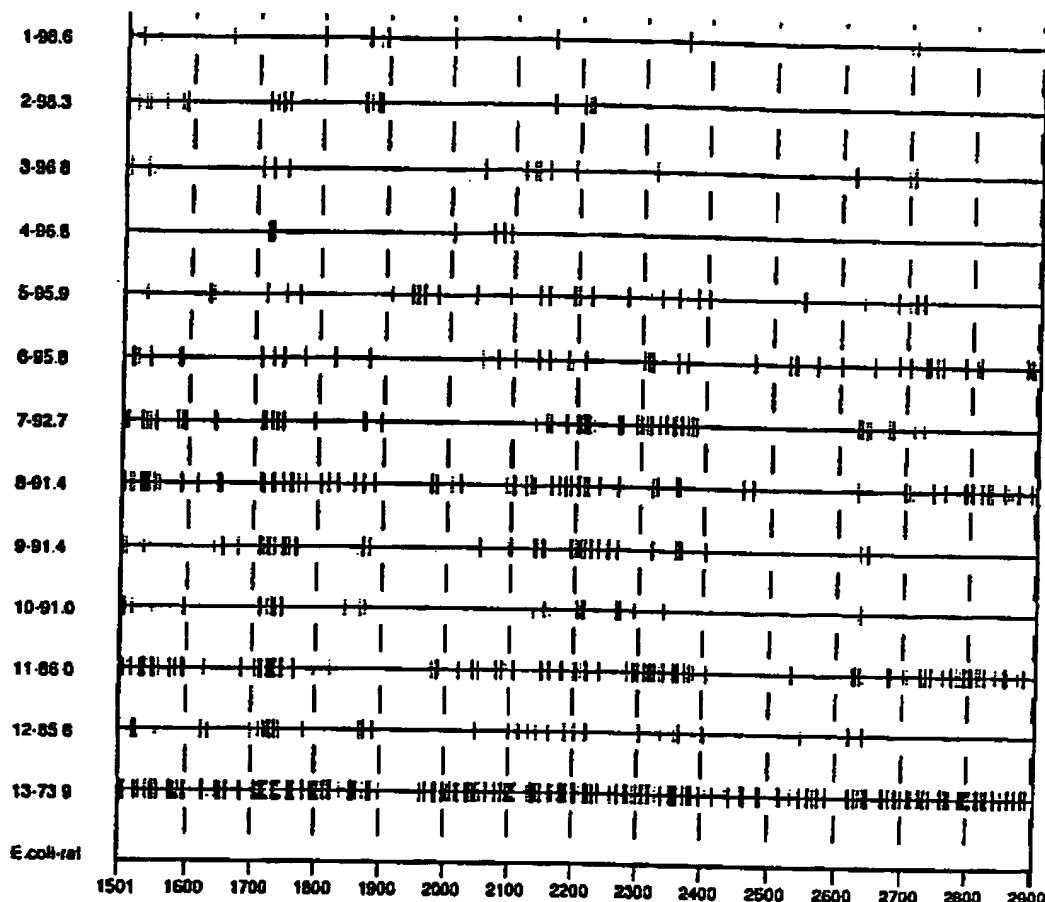
FIG. 7  
Summary of 23S rRNA Analysis



LEGEND: SUMMARY of 23S rRNA Analysis (Listing of Bacteria and percent similarity included in analysis)

1. 98.6% *Neisseria gonorrhoeae*-*Neisseria meningitidis*;
2. 98.3% *Proteus mirabilis*-*Proteus vulgaris*;
3. 96.8% *Mycobacterium intracellulare*-*Mycobacterium avium*;
4. 96.8% *Mycobacterium avium*-*Mycobacterium asiaticum*;
5. 95.9% *Mycobacterium tuberculosis*-*Mycobacterium kansasii*;
6. 95.8% *Nicotiana tabacum* (tobacco)-*Zea mays* (maize);
7. 92.7% *Proteus vulgaris*-*Klebsiella rhinoscleromatis*;
8. 91.4% *Bacillus stearothermophilus*-*Bacillus subtilis*;
9. 91.4% *Mycobacterium intracellulare*-*Mycobacterium fortuitum*;
10. 91.0% *Escherichia coli*-*Klebsiella rhinoscleromatis*;
11. 86.0% *Escherichia coli*-*Pseudomonas aeruginosa*;
12. 85.8% *Chlamydia trachomatis*-*Chlamydia psittaci*;
13. 73.9% *Escherichia coli*-*Anacystis nidulans*.

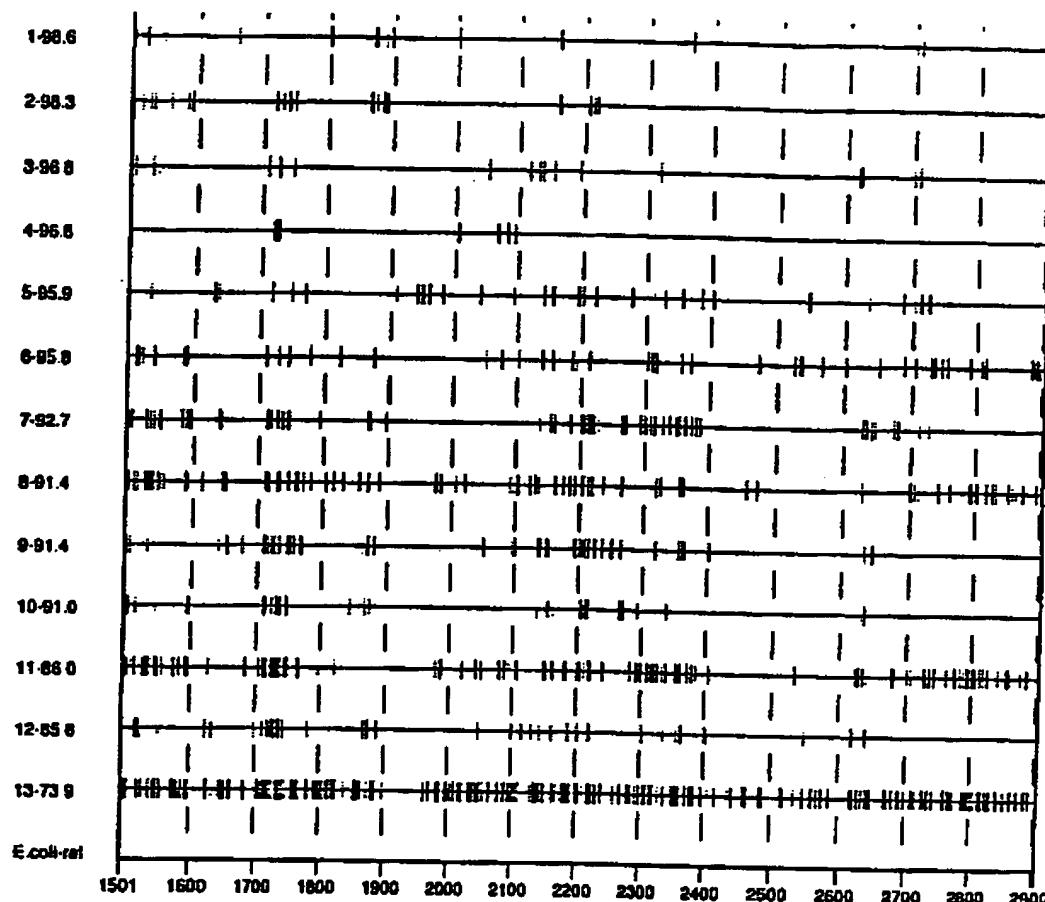
FIG. 8  
Summary of 23S rRNA Analysis



LEGEND: SUMMARY of 23S rRNA Analysis (Listing of Bacteria and percent similarity included in analysis)

1. 98.6% *Neisseria gonorrhoeae*-*Neisseria meningitidis*;
2. 98.3% *Proteus mirabilis*-*Proteus vulgaris*;
3. 96.8% *Mycobacterium intracellulare*-*Mycobacterium avium*;
4. 96.8% *Mycobacterium avium*-*Mycobacterium asiaticum*;
5. 95.9% *Mycobacterium tuberculosis*-*Mycobacterium kansasii*;
6. 95.8% *Nicotiana tabacum* (tobacco)-*Zea mays* (maize);
7. 92.7% *Proteus vulgaris*-*Klebsiella rhinoscleromatis*;
8. 91.4% *Bacillus stearothermophilus*-*Bacillus subtilis*;
9. 91.4% *Mycobacterium intracellulare*-*Mycobacterium tortuitum*;
10. 91.0% *Escherichia coli*-*Klebsiella rhinoscleromatis*;
11. 86.0% *Escherichia coli*-*Pseudomonas aeruginosa*;
12. 85.8% *Chlamydia trachomatis*-*Chlamydia psittaci*;
13. 73.9% *Escherichia coli*-*Anacystis nidulans*.

FIG. 8  
Summary of 23S rRNA Analysis



LEGEND: SUMMARY of 23S rRNA Analysis (Listing of Bacteria and percent similarity included in analysis)

1. 98.6% *Neisseria gonorrhoeae*-*Neisseria meningitidis*;
2. 98.3% *Proteus mirabilis*-*Proteus vulgaris*;
3. 96.8% *Mycobacterium intracellulare*-*Mycobacterium avium*;
4. 96.8% *Mycobacterium avium*-*Mycobacterium asiaticum*;
5. 95.9% *Mycobacterium tuberculosis*-*Mycobacterium kansasii*;
6. 95.8% *Nicotiana tabacum* (tobacco)-*Zea mays* (maize);
7. 92.7% *Proteus vulgaris*-*Klebsiella rhinoscleromatis*;
8. 91.4% *Bacillus stearothermophilus*-*Bacillus subtilis*;
9. 91.4% *Mycobacterium intracellulare*-*Mycobacterium tortuitum*;
10. 91.0% *Escherichia coli*-*Klebsiella rhinoscleromatis*;
11. 86.0% *Escherichia coli*-*Pseudomonas aeruginosa*;
12. 85.8% *Chlamydia trachomatis*-*Chlamydia psittaci*;
13. 73.9% *Escherichia coli*-*Anacystis nidulans*.

## Summary of 16S rRNA PROBE locations

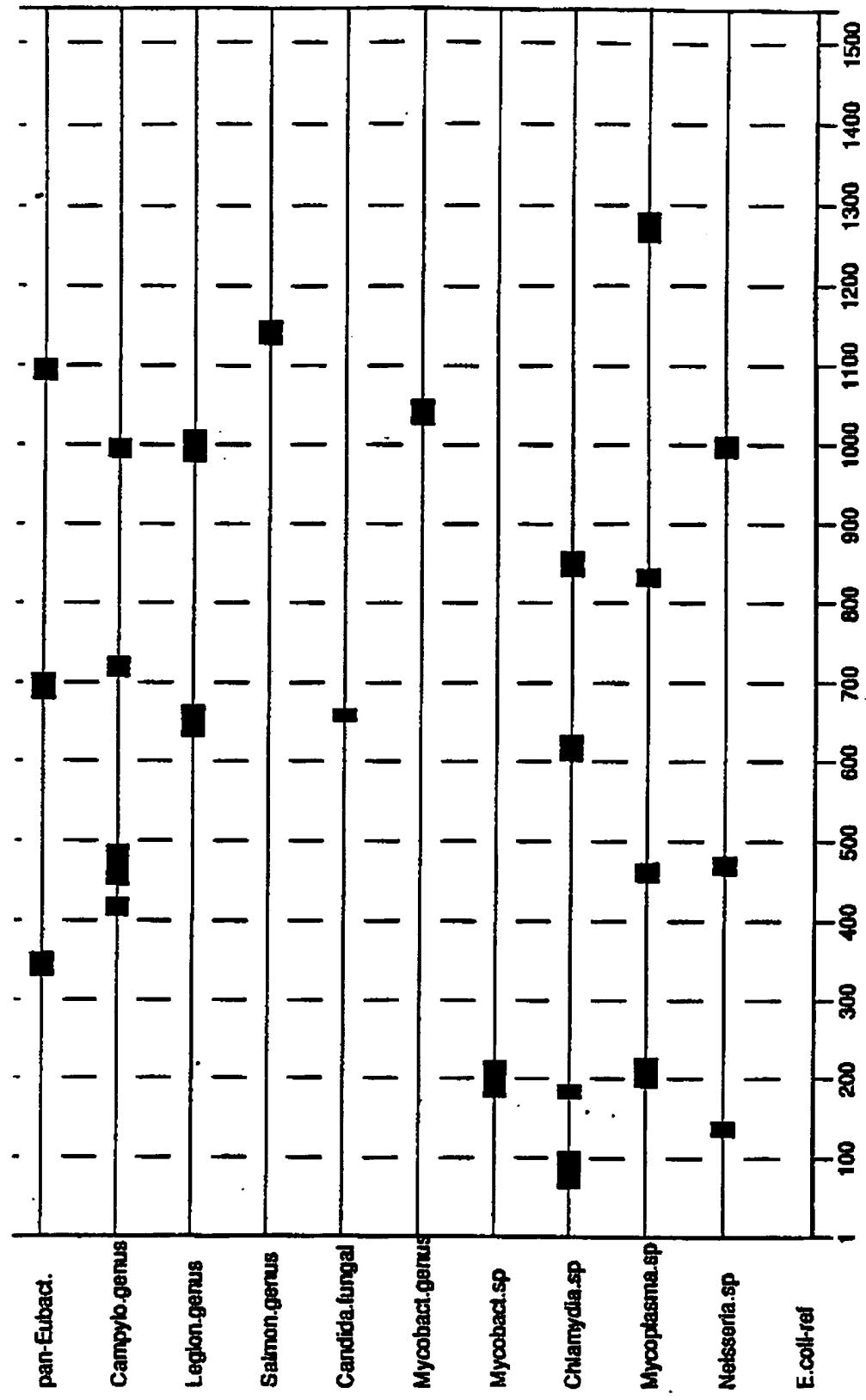


FIG. 9

### Summary of 23S rRNA PROBE locations

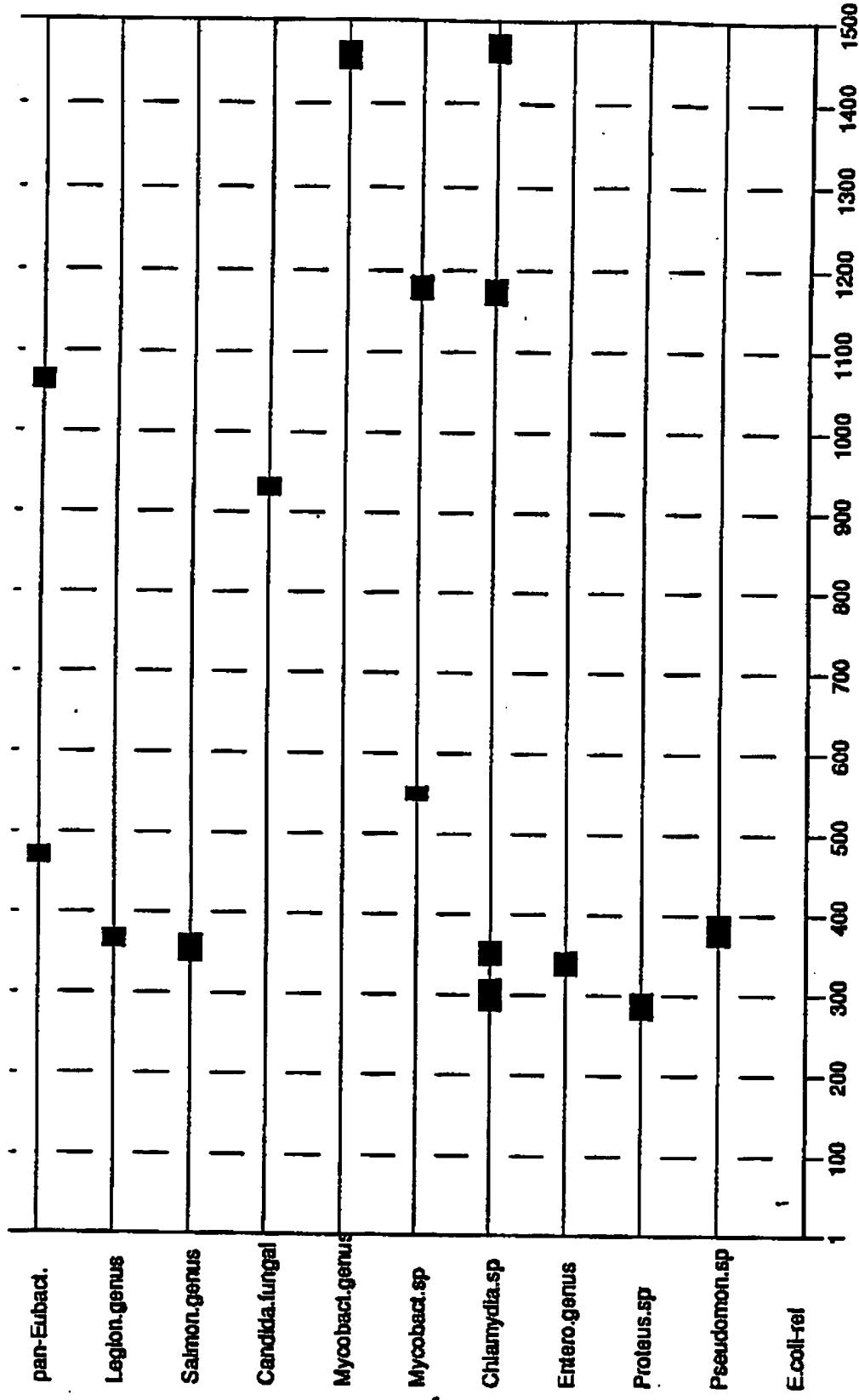


FIG. 10

### Summary of 23S rRNA PROBE locations

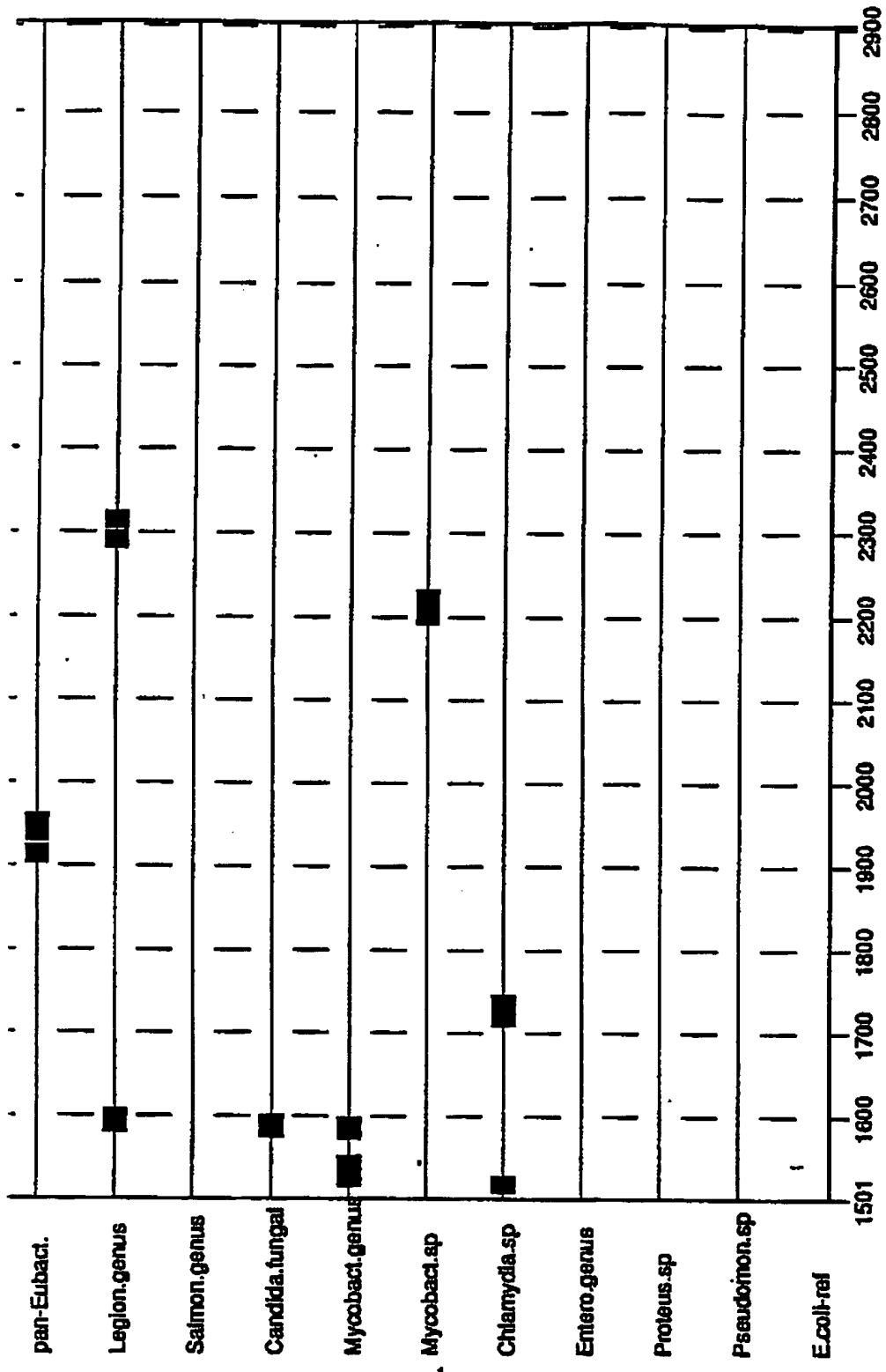


FIG. 11